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NAME OF SUPERVISOR/*NOM DU DIRECTEUR DE THÈSE* Dr. W. A. AYER

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THE UNIVERSITY OF ALBERTA

I. STRUCTURAL STUDIES ON SOME *LYCOPODIUM ALKALOIDS*

II. AN INVESTIGATION OF THE SWIMMING RESPONSE OF THE SEA ANEMONE *STOMPHIA COCCINEA* TO CERTAIN STARFISH

by

PETER SINGER ©

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA
FALL, 1975

THE UNIVERSITY OF ALBERTA
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled

I. STRUCTURAL STUDIES ON SOME *LYCOPODIUM* ALKALOIDS

II. AN INVESTIGATION OF THE SWIMMING RESPONSE OF THE SEA ANEMONE *STOMPHIA COCCINEA* TO CERTAIN STARFISH

submitted by PETER SINGER in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

W.A. Ayer
W. A. Ayer
Supervisor

Date: 26 Sep 1975

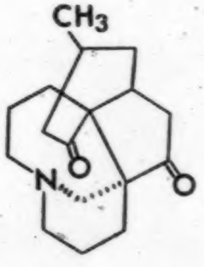
ABSTRACT

We have investigated the basic components of two species of Lycopodium. L. alpinum contains the alkaloids lycopodine, clavolonine, lycodine, des-N-methyl-α-obscurine and a new alkaloid, lycoflexine. L. clavatum var. inflexum contains the alkaloids lycopodine, dihydrolycopodine, lycodoline, fawcettimine and two new alkaloids, lycoflexine and 6-hydroxylycopodine lactam.

This thesis describes the chemical and physical methods used to derive the structure of 6-hydroxylycopodine lactam (24), the first reported lactam alkaloid isolated from a Lycopodium species. In addition, chemical and physical studies towards the structure elucidation of lycoflexine (13), are reported. The complete structure of lycoflexine, a new type of Lycopodium alkaloid, was established by x-ray crystallographic studies. Most Lycopodium alkaloids have a C_{16} skeleton; biogenetic implications of this new C_{17} skeleton are discussed.

The use of carbon 13 n.m.r. (c.m.r.) as a physical method for the structure determination of the Lycopodium alkaloids was investigated. The c.m.r. spectra were determined for eight alkaloids of the lycopodine, (1), skeletal type and four alkaloids of the lycodine, (3), skeletal type. The n.m.r. resonances were assigned for each carbon nucleus, changes in the position of resonance being correlated with structural changes. The c.m.r. assignments were used to verify that sauroxine, (34), is the C-12 epimer of α-obscurine, (33). Interestingly, the results showed that the N-methyl group of N-methyllycodine, sauroxine and α-obscurine are axial.

The sea anemone Stomphia coccinea will detach and swim when contact is made with the starfish Dermasterias imbricata. The second part of this thesis describes the isolation and attempted purification of the substance eliciting the swimming response. Analysis of Dermasterias extracts shows the presence of steroids. However, the compound inducing the swimming response is not steroidal, but an acid and base sensitive water soluble anion. Purification of the compound is difficult but evidence is presented which indicates that it may be a nucleotide-type compound.



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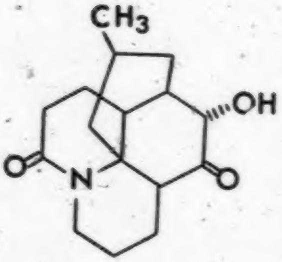

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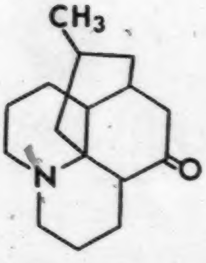

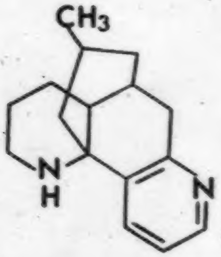

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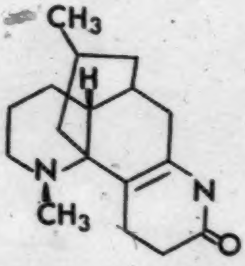

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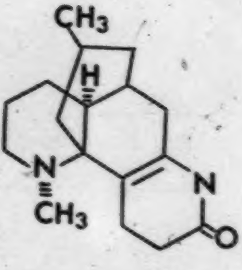

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I : STRUCTURAL STUDIES ON SOME *LYCOPODIUM* ALKALOIDS

INTRODUCTION

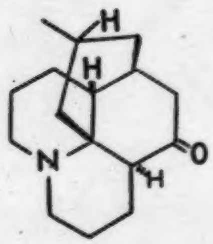
Since the isolation of lycopodine in 1881 from *Lycopodium complanatum* over one hundred alkaloids have been isolated from club mosses of the *Lycopodium* family. About twenty-five of the more than two hundred species of *Lycopodium* have been investigated and the work has been extensively reviewed[1].

In the late 1950's and early 1960's the structures of the *Lycopodium* alkaloids began to be clarified[2]. Based on their carbon-nitrogen skeleton, the alkaloids of known structure can be divided into nine structural groups (Chart I): the lycopodine, annotinine, lycodine, annotine, cernuine, serratinine, annopodine, alopecurine, and luciduline groups.

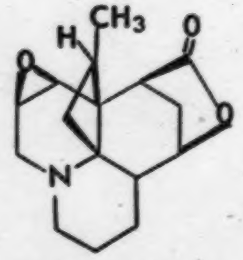
Two species of *Lycopodium* were investigated for their alkaloid content. Work initially began on *Lycopodium alpinum* and a new alkaloid was found, later named lycoflexine, $C_{17}H_{25}NO_2$, m.p. 130-131°C. Subsequently the same alkaloid was found in *Lycopodium clavatum* var. *inflexum* and this species was also examined.

The molecular formula and the mass spectrum of

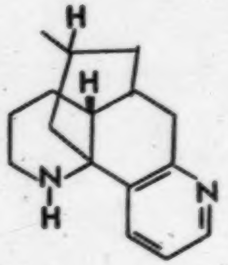
CHART 1. Structural types of the Lycopodium alkaloids



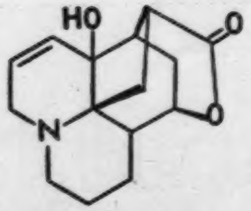
Lycopodine (1)



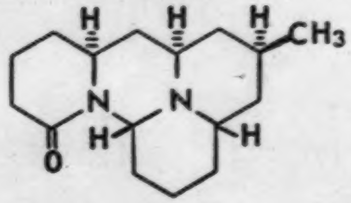
Annotinine (2)



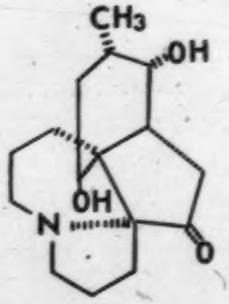
Lycodine (3)



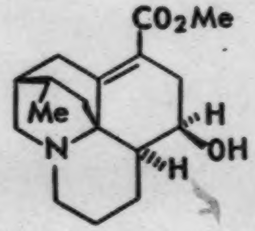
Annotine (4)



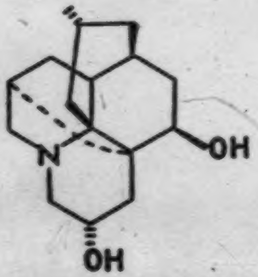
Cernuine (5)



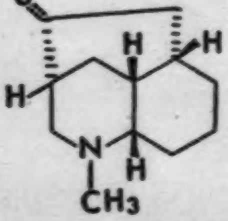
Serratinine (6)



Annopodine (7)



Alopecurine (8)



Luciduline (9)

lycoflexine indicated a new type of carbon-nitrogen skeleton and this thesis describes the investigation leading to the structure determination of lycoflexine.

Of the known Lycopodium alkaloids most are C_{16} compounds. The first eight of the nine groups have a basic C_{16} skeleton. The ninth i.e. luciduline is a C_{13} compound. In addition, nicotine, $C_{10}H_{14}N_2$, and several other compounds with fewer than sixteen carbon atoms have been isolated[3]. In cases where there are more than sixteen carbons the extra carbons are due to the formation of simple derivatives e.g. N-methyl and N- or O- acetyl compounds. Lycoflexine with seventeen carbons appeared not to be such a simple derivative and so there was greater interest in its structure determination.

The mass spectrum of lycoflexine is dominated by the mass peak m/e 275 and the base peak m/e 84, corresponding to $C_{12}H_{25}NO_2$ and $C_5H_{10}N$ respectively.

The biosynthesis of the Lycopodium alkaloids is not yet resolved.* However it is known that for the C_{16} alkaloids eight of the carbon atoms are derived from a "pelletierine unit" and the other eight from pelletierine precursors (lysine and acetate) Fig. I[4]. Cadaverine (10) and

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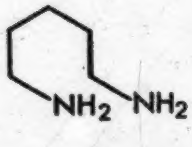
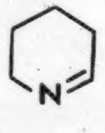
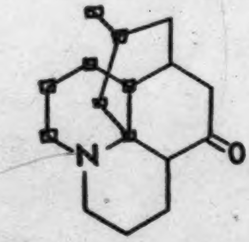
* Recently W. D. Marshall et. al. Can. J. Chem. 53, 41 (1975) have elaborated on the biosynthesis.



Cadaverine (10)



Δ^1–Piperideine (11)



□ Carbons derived from Pelletierine

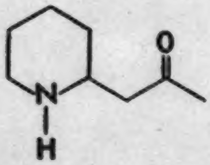


Fig. 1. Biogenesis of lycopodine.

Δ^1-piperideine (11) are also incorporated into lycopodine (taking the place of lysine)[5]. As mentioned lycoflexine has an apparently simple fragmentation to a $C_5H_{10}N$ unit. Hence there was a greater interest in the determination of the lycoflexine structure as both these unusual features, *i.e.*, the origin of the seventeenth carbon atom and the origin of a $C_5H_{10}N$ unit might suggest a biosynthetic pathway in the *Lycopodium* alkaloids.

A second alkaloid, $C_{16}H_{23}NO_3$, m.p. 200-202°C, was isolated from *Lycopodium* *clavatum* var. *inflexum* and initial spectral analysis indicated a new alkaloid of the lycopodine group. The chemical and physical studies towards the elucidation of its structure are described.

In the course of this work the carbon 13 n.m.r. spectra of lycopodine and several other *Lycopodium* alkaloids were extensively investigated and the resonances assigned. Since early work in the 1950's with carbon 13 n.m.r.[6], rapid progress has been made in instrumentation, initially with computer averaging of a large number of continuous wave scans and more recently with pulse irradiation and the use of a computer to perform Fourier transform operations to obtain the spectra. This latter method is now the preferred one. The sample is irradiated with a large number of pulses in a relatively short time enabling the carbon 13 n.m.r. spectra of quite small samples (e.g. half a millimole) to be obtained[7]. With these advances, carbon 13 n.m.r. is a useful addition to the physical methods available for structure elucidation and has been used for this purpose[8]. The carbon 13 n.m.r. spectra of the Lycopodium alkaloids were obtained in order to examine the possibility of using carbon 13 n.m.r. as a tool towards the structure elucidation of lycoflexine and other alkaloids. The results of this work are described.

DISCUSSION AND RESULTS

[A] The Structure of Lycoflexine

Lycopodium alpinum was collected from the Jasper area of the Canadian Rocky Mountains where it grows around the tree line at about 7,000 ft. A preliminary investigation* had yielded a minor alkaloid with the unusual peaks m/e 275 and m/e 84 in the mass spectrum and a more detailed investigation was thus initiated.

The plant was freed from earth, moss, etc. and allowed to dry in the air for one week. The dried material was ground to a powder and extracted in a Soxhlet apparatus with methanol for seventy-two hours. The methanol solution was concentrated under reduced pressure until a thick sludge was obtained. This was then heated under reflux in ethyl acetate containing a small amount of concentrated ammonium hydroxide solution. The yellow solution and the residue were extracted with hydrochloric acid and this aqueous solution was basified with ammonia to pH 9.5. The basic solution was extracted with chloroform to give the crude alkaloid mixture.

---

* By E. Bertram, Senior honors project (Chem. 501), 1965.

This procedure was inefficient, yielding only 3.7 g. from 4.5 kg. of dried plant, and subsequently the methanolic sludge was taken up in hydrochloric acid, extracted with ether to remove neutral compounds and basified to pH 11 with concentrated sodium hydroxide solution. Immediate extraction of this solution with chloroform gave the crude alkaloid mixture in greater yield.

The crude alkaloid mixture was chromatographed over alumina, eluting with benzene, ether, chloroform, methanol and mixtures of these solvents. Crystallization of these fractions led to the isolation of lycopodine and clavolonine as the major alkaloids. Smaller amounts of lycodine and des-N-methyl-α-obscurine were also obtained. In all cases identity was established by direct comparison with authentic samples. The desired compound, subsequently named lycoflexine, which was present in only very small quantities, was eluted with chloroform.

At about this time Dr. B. Altenkirk of the South African Medical Research Council, forwarded to us a sample of an alkaloid which he had isolated from a species of Lycopodium (L. clavatum var. inflexum) which occurs in the Transvaal. This alkaloid also showed the characteristic peaks at m/e 275 and m/e 84 in the mass spectrum. Further investigation revealed its identity with the L. alpinum alkaloid. Lycoflexine occurs to a larger extent in L. clavatum var. inflexum than in L. alpinum and is easier to separate from this species. With the cooperation of Dr. Altenkirk it was decided to pursue the structure of this apparently novel substance using the South African species as the alkaloid source. The name lycoflexine was derived from this source.

The total methanol extract of the plant was obtained from S. Africa and the crude alkaloid mixture was obtained in the same way as from L. alpinum. The alkaloid mixture was chromatographed over alumina. Repeated column chromatography of the chloroform fraction yielded pure lycoflexine which crystallized on evaporation of the solvent. The alkaloid mixture contained about 3.5% lycoflexine.

Lycoflexine, m.p. 130-131°, forms a perchlorate salt, m.p. 200-201°, when treated with perchloric acid in acetone. The i.r. spectrum ($CHCl_3$) of lycoflexine (Fig. 2), indicates the absence of O-H and N-H functional groups but shows carbonyl bands at 1695 cm^{-1} and 1725 cm^{-1}. In the presence of traces of acid in the chloroform the band at 1725 cm^{-1} shifts to higher wavenumber, 1740 cm^{-1}, and a broad band appears at 2300 cm^{-1} characteristic of $-\overset{|\bullet}{\underset{|}{N}}H$ In lycoflexine perchlorate this carbonyl band appears at 1745 cm^{-1}, while a nujol mull spectrum of the free base shows bands at 1695 cm^{-1} and 1720 cm^{-1}. There is no evidence of $>N-CH_3$ absorption in the spectrum but weak bands at 1410 cm^{-1} reveal the presence of active methylene.

The mass spectrum (Fig. 3) is particularly interesting. The mass peak at m/e 275 (65%) fragments to the base peak at m/e 84 and by high resolution mass spectroscopy these ions were found to correspondend to $C_{17}H_{25}NO_2$ and $C_5H_{10}N$ respectively. The molecule also loses CO to give a peak at m/e 247 (26%) corresponding to $C_{16}H_{25}NO$.

The mass spectrum of lycopodine indicates that fragmentation involves loss of the "bridging" carbons (and any substituents on them) as in Fig. 4[9].

The loss of 57 (or 73 for clavolonine) dominates the spectrum of this skeletal type. Its complete absence in the mass spectrum of lycoflexine indicated that it did not possess the lycopodine skeleton. Furthermore, the mass spectral fragmentation pattern of lycoflexine bears no relationship to that of any of the known *Lycopodium*

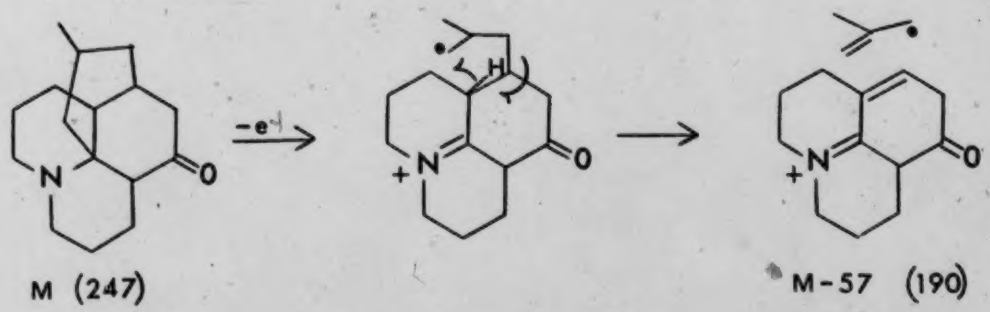
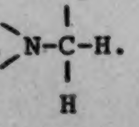


Fig. 4. The mass spectral fragmentation of lycopodine.

structural groups and indicated a new skeletal type.

The u.v. spectrum shows absorption at λ_{max} 230 nm, (ε 1380) and 298 nm (ε 220).

The p.m.r. spectrum is shown in Fig. 5. The doublet at δ 0.95 (J=6c.p.s.) clearly shows the presence of a $CH-CH_3$ unit. There are no signals for olefinic protons. The low field protons, i.e., the signals at δ 3.62, 3.48, 3.20, 2.78 and 2.64, were attributed to hydrogens on carbons α to the nitrogen. The δ 3.62, 3.48, 2.78 and 2.64 signals appeared as an AB system. Irradiation of one of the protons caused the signal of the other to collapse showing that they were coupled. The large coupling constant (J=14c.p.s.) showed it to be a geminal coupling, i.e.



$>N-\underset{H}{\overset{|}{\underset{|}{C}}}-H$.

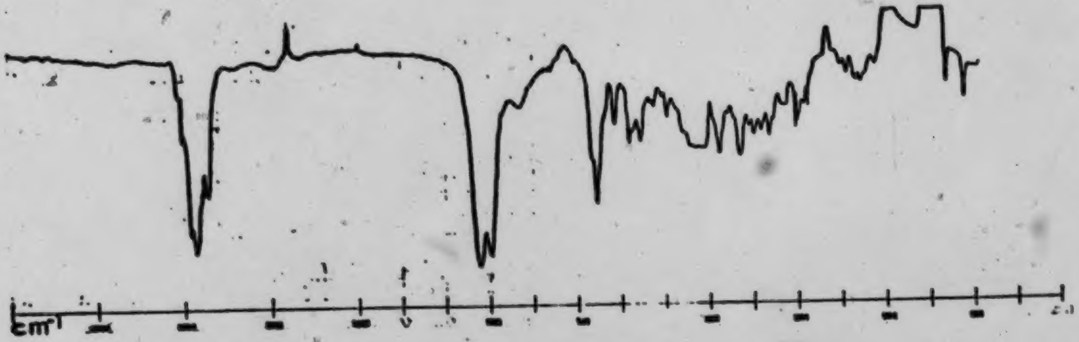


Fig. 2. The i. r. spectrum of lycoflexine.

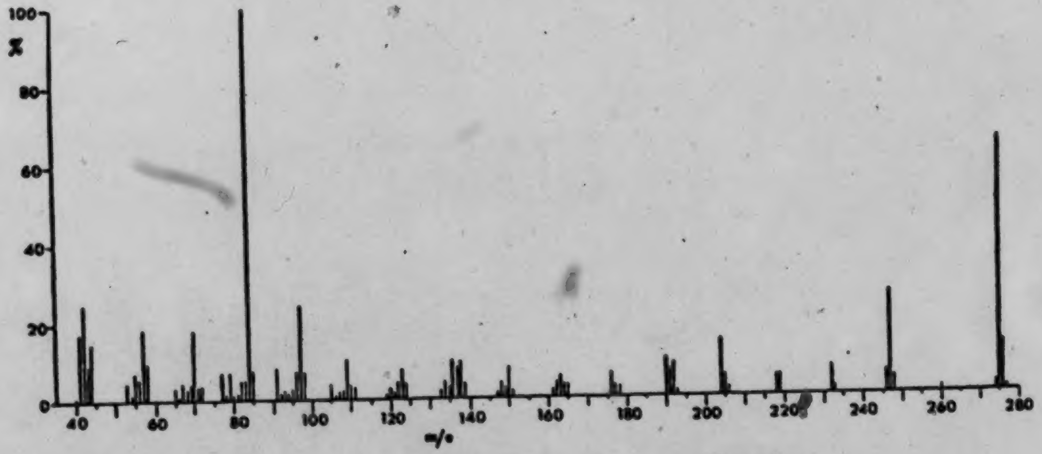
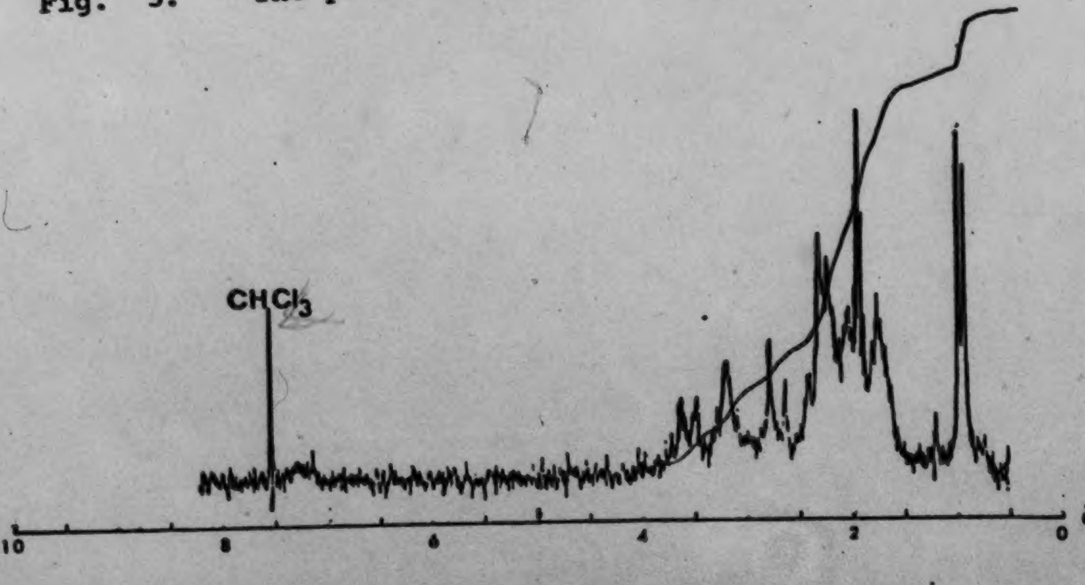
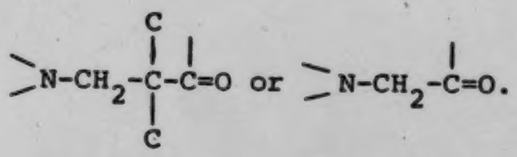


Fig. 3. The mass spectrum of lycoflexine.

Fig. 5. The p. m. r. spectrum of lycoflexine.



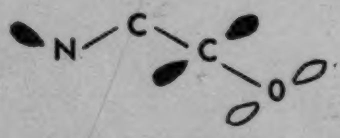
Further splitting of these signals is small and probably results from long range coupling, compatible with either



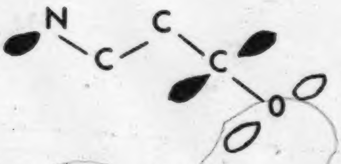
$$>N\text{-}CH_2\text{-}\underset{\underset{C}{|}}{\overset{\overset{C}{|}}{C}}\text{-}\overset{|}{C}{=}O \text{ or } >N\text{-}CH_2\text{-}\overset{|}{C}{=}O.$$

The large difference in shift between the signals centred at δ 3.55 and δ 2.71 may be accounted for by one of the protons being affected by the carbonyl group. Decoupling the methyl doublet showed the resonance of the methine is at δ 2.24.

The shift of the carbonyl absorption in the i.r. spectrum on protonation indicated some interaction between the nitrogen and the carbonyl group. This suggests the partial structure $>N\text{-}\underset{|}{\overset{|}{C}}\text{-}\overset{|}{C}{=}O$ or $>N\text{-}\underset{|}{\overset{|}{C}}\text{-}\underset{|}{\overset{|}{C}}\text{-}\overset{|}{C}{=}O$. With these partial structures there is the possibility of "W" coupling[10]:



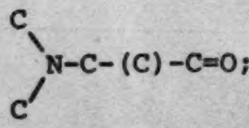
or an interaction transmitted via σ bonds when the nitrogen lone pair, the carbon-carbon bond and the carbonyl π bond are all parallel[10]:



In view of this possible interaction the o.r.d. and c.d. spectra of lycoflexine were obtained, (Fig. 6). The c.d. spectrum shows three absorptions (326, 298 and 240 nm). Since there are only two carbonyl groups in the molecule the third absorption is indicative of a "non-classical" interaction.

Acidifying the solution protonates the nitrogen and the lone pair is no longer free. The c.d. and o.r.d. spectra of the acidified solution (Fig. 7) show the loss of the "non-classical" absorption band, and only the two carbonyl n→π^* absorptions are seen. In addition the 298 nm band is now positive whereas in the free base it is negative.

This confirms the presence of a nitrogen lone pair-carbonyl interaction but does not enable the distinction to be made between the α- and β-amino ketone.

So far the spectral analyses suggest the partial structures:  $\mathrm{(C)(C)N{-}C{-}(C){-}C{=}O}$; C=O; $\mathrm{CH{-}CH_3}$ in a tetracyclic

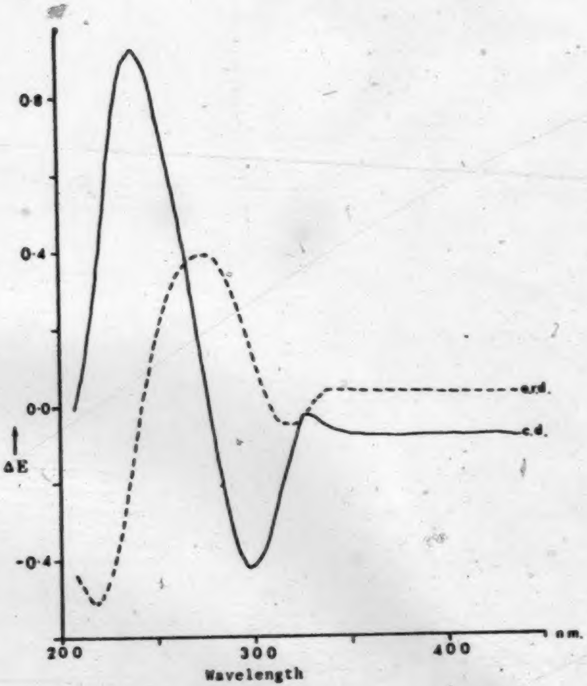


Fig. 6. The c. d. and o. r. d. spectra of lycoflexine.

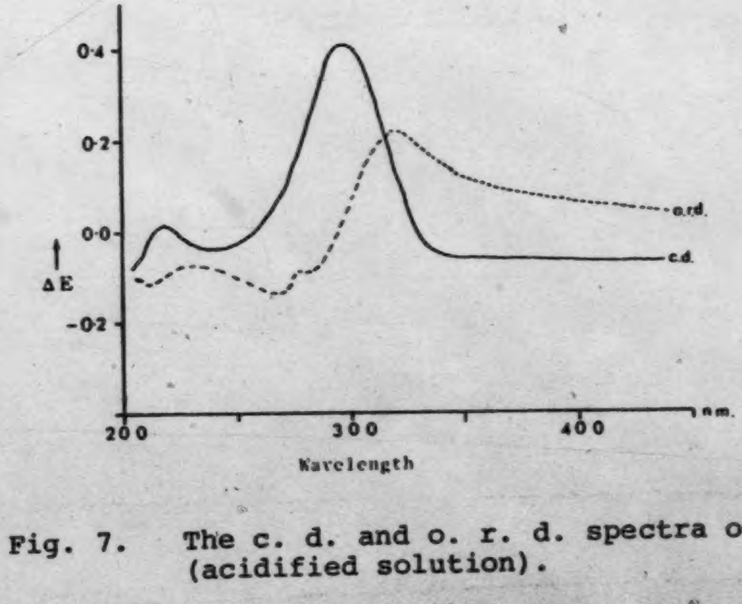


Fig. 7. The c. d. and o. r. d. spectra of lycoflexine (acidified solution).

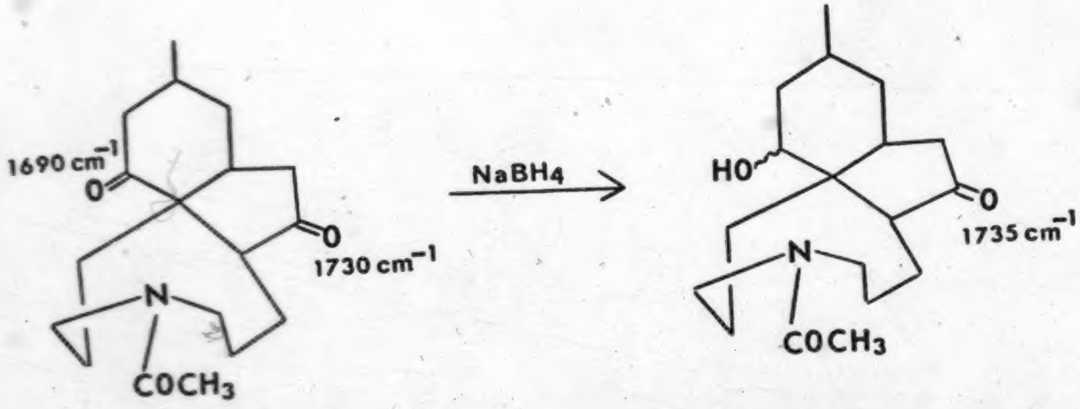
skeleton.

Treatment of lycoflexine with D_2O produced no change in its n.m.r. spectra and attempted acetylation with acetic anhydride (pyridine catalyst) likewise produced no change. These results confirm the absence of N-H and O-H functional groups.

Reduction of lycoflexine with sodium borohydride produces a ketoalcohol. The i.r. spectrum of the ketoalcohol shows that the carbonyl absorbing at 1695 cm^{-1} in the i.r. spectrum of lycoflexine is reduced, while the carbonyl absorbing at 1725 cm^{-1} is unaffected, possibly because the interaction with the nitrogen lone pair reduces its electrophilicity. However N-acetylfawcettimine[11] shows similar behavior. In this case selective reduction of a cyclohexanone in the presence of a cyclopentanone is observed.

Implicit in this comparison is the conclusion that lycoflexine contains a six-membered ketone (1695 cm^{-1}) and a five-membered ketone (1725 cm^{-1}).

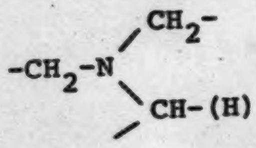
Treatment of lycoflexine with methyl iodide in acetone produced a crystalline methiodide salt which was



N-acetylfawcettimine

dihydroxy-N-acetylfawcettimine

recrystallized from chloroform. Lycoflexine methiodide $C_{17}H_{25}NO_2 \cdot CH_3I$, m.p. 243-244°, shows a sharp 3-proton methyl signal in the p.m.r. spectrum (Fig. 8) at δ 3.12. This confirms the absence of an N-methyl group in lycoflexine itself. Using the N-methyl signal as an internal standard for three protons, the integration of the low field region indicates five to six protons (other than $>N-CH_3$), α to the nitrogen. Thus lycoflexine contains the structural unit


$-CH_2-N\begin{matrix} \diagup CH_2- \\ \diagdown CH-(H) \\ \diagup \end{matrix}$.

It was hoped that Hofmann degradation of the methiodide would yield more structural information. Lycoflexine methiodide was heated under reflux with sodium t-butoxide in t-butyl alcohol for four hours. The yield of the reaction was low (less than 25%). Thin layer chromatography revealed one major product together with three minor ones and no starting material. The mass spectrum of the product mixture shows major peaks at m/e 289(17%); 248(60%); 220(21%); 205(18%); 177(38%); 139(100%); and 71(15%) (Fig. 9). The m/e 289 peak had the composition $C_{18}H_{27}NO_2$ by exact mass determination. The fragmentation leading to an m/e 84 peak (in lycoflexine) was no longer prominent.

This indicated that Hofmann elimination had occurred. The reaction was repeated on a larger scale, this time with a slightly higher base concentration and a longer reaction time. The product of this reaction, which could be recrystallized from acetone (m.p. 168-170°), was more polar (by t.l.c.) than the previously obtained material. Its mass spectrum shows peaks at m/e 303(44%); 287(85%); 262(94%); 198(25%); 124(100%); 71(88%) and 70(85%) (Fig. 10). This product, however, proved to be very unstable, decomposing in a few days and before exact mass determinations had been secured. Before it had decomposed an i.r. spectrum was obtained (Fig. 11) which showed a sharp band at 3500 cm^{-1}, indicating the presence of a hydroxyl group, a shoulder at 1700 cm^{-1} and carbonyl bands at 1715 cm^{-1} and 1675 cm^{-1}. A fairly weak band at 2800 cm^{-1} is indicative of an N-methyl group.

Fig. 8.

The p. m. r. spectrum of lycoflexine methiodide.

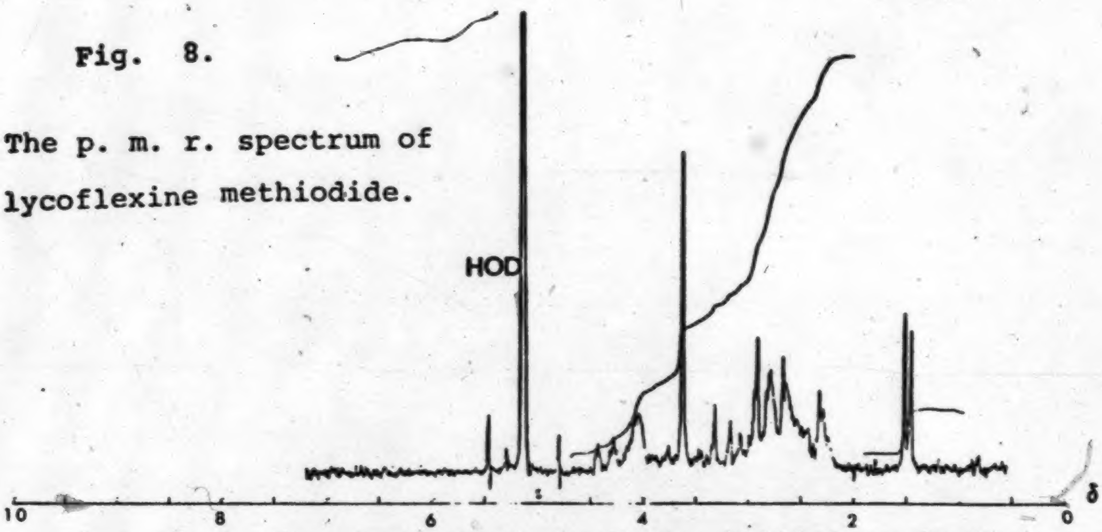


Fig. 9. The mass spectrum of the initial product of the Hofmann elimination reaction of lycoflexine methiodide.

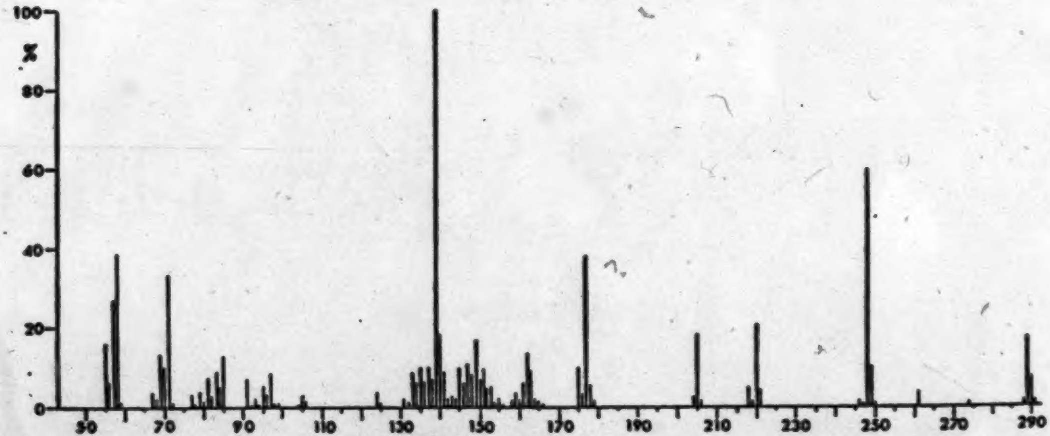
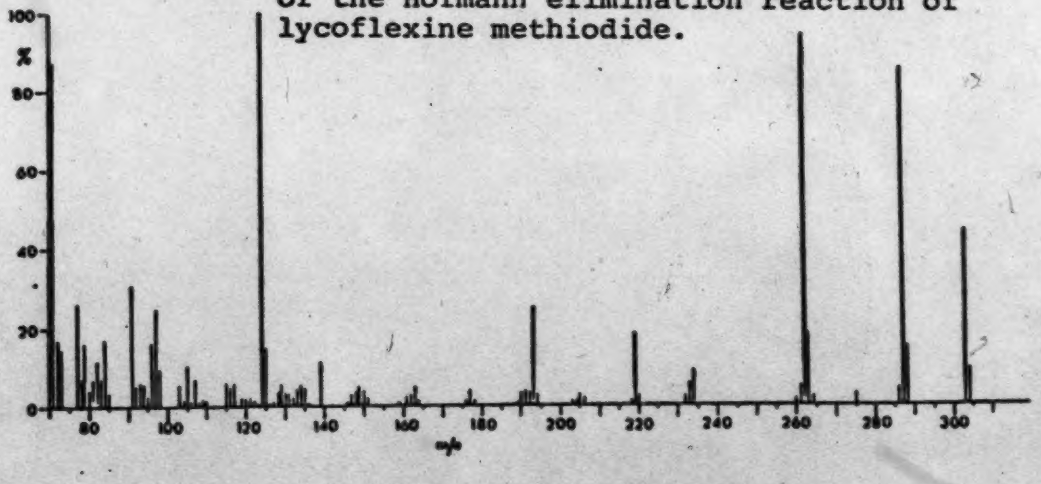


Fig. 10. The mass spectrum of the final product of the Hofmann elimination reaction of lycoflexine methiodide.



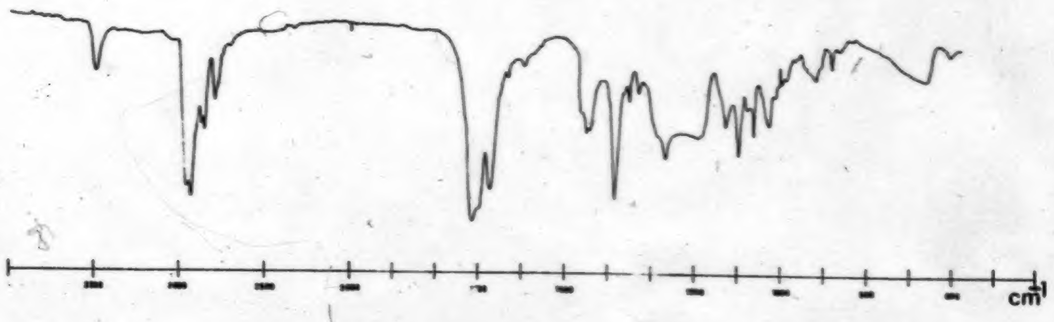


Fig. 11. The i. r. spectrum of the final product of the Hofmann elimination reaction of lycoflexine methiodide.

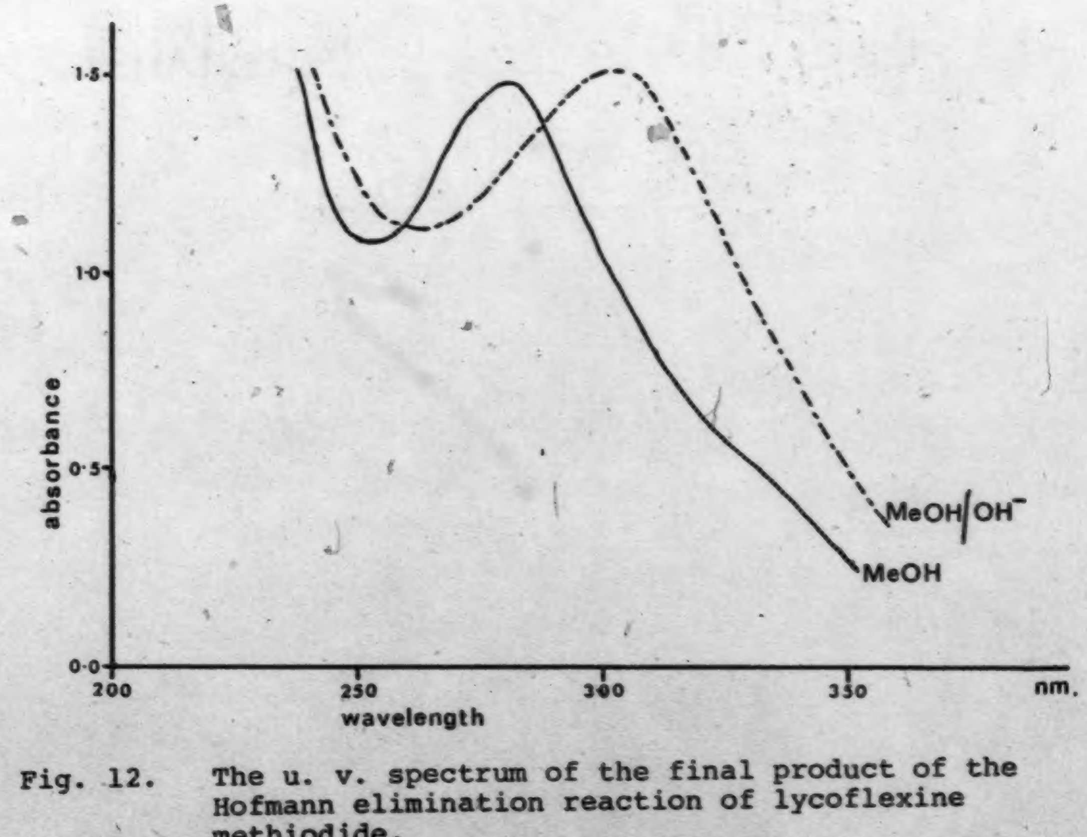
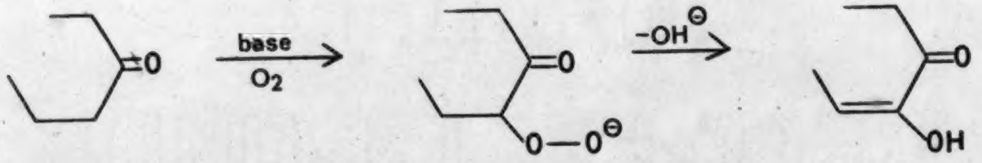


Fig. 12. The u. v. spectrum of the final product of the Hofmann elimination reaction of lycoflexine methiodide.

The u.v. spectrum (Fig. 12) of this compound has λ_{max} 280 nm. (ε 5,600) in methanol. Addition of one drop of sodium hydroxide solution shifts the absorption to λ_{max} 293 nm. (ε 8,900).

This spectral information suggests that a second reaction took place in addition to the Hofmann reaction:



The mass spectrum of the reaction product has a molecular ion at m/e 303. This is equivalent to addition of O and loss of 2H, *i.e.* an addition of 14 mass units over that of lycoflexine methiodide cation. The u.v. data compares favourably with that for diosphenols. This would explain the sharp band in the i.r. spectrum at 3500 cm^{-1} (intramolecular hydrogen bonded OH) and if a portion is in the diketone form would account for the shoulder on the carbonyl band. The absorptions of the carbonyls indicate that the one occurring at 1725 cm^{-1} does not participate in the reaction and the one at 1695 cm^{-1} became conjugated. Thus all the spectral information indicates that lycoflexine methiodide is oxidized by molecular oxygen in basic solution[12].

To test this hypothesis lycoflexine itself was heated under reflux in t-butyl alcohol with sodium t-butoxide in a nitrogen atmosphere. No reaction occurred. Repeating the reaction but this time bubbling oxygen through the reaction mixture produced a new product within two hours (as shown by t.l.c.). The reaction was repeated on a larger scale; the reaction did not proceed to completion, the yield was low and the product unstable. However the product could be purified by chromatography over alumina (product eluted with 1-propanol) to give a non-crystalline material, which decomposes on heating.

This product has a mass spectrum (Fig. 13) with peaks at m/e 289(100%), corresponding to $C_{17}H_{23}NO_3$; m/e 272(15%, M-17); 261(20%) corresponding to both $C_{15}H_{19}NO_3$ and $C_{16}H_{23}NO_2$; 206(35%); 163(25%) and 79(39%). It thus appears that lycoflexine can be oxidized under these conditions to give a diosphenol. Once again the m/e 84 peak, which dominates the mass spectrum of lycoflexine, is not now present and m/e 206 is a major fragment.

Although this compound might have been very useful in further degradation, the difficulty in obtaining it and its instability discouraged further study, particularly since our supply of lycoflexine was limited.

The unexpected results from the Hofmann reaction can now be explained. Lycoflexine has molecular weight 275; the cation in lycoflexine methiodide 290. The Hofmann elimination should give a compound with mass 289 and subsequent oxidation would give a compound of mass 303, as is the case.

A procedure successfully used for deuteration of lycopodine was used to investigate the number of enolizable hydrogens in lycoflexine. Lycoflexine was shaken in deuterium oxide with phosphorus pentachloride and the solution left at room temperature for several days. Neutralization yielded the deuterated lycoflexine. The mass spectrum shows this to be a mixture of $C_{17}H_{25-n}NO_2D_n$ $(n=0 \rightarrow 5)$. The amount of each was in the ratio m/e 275/1; 276/3; 277/8; 278/9; 279/4.5; 280/1. The peak corresponding to $C_{17}H_{20}NO_2D_5$ was small and possibly an isotope peak. However, an exact mass determination of the 280 peak confirmed it was that of $C_{17}H_{20}NO_2D_5$. Hence there are five hydrogens replaceable by deuterium, suggesting the possibility of five hydrogens α to the carbonyl groups.

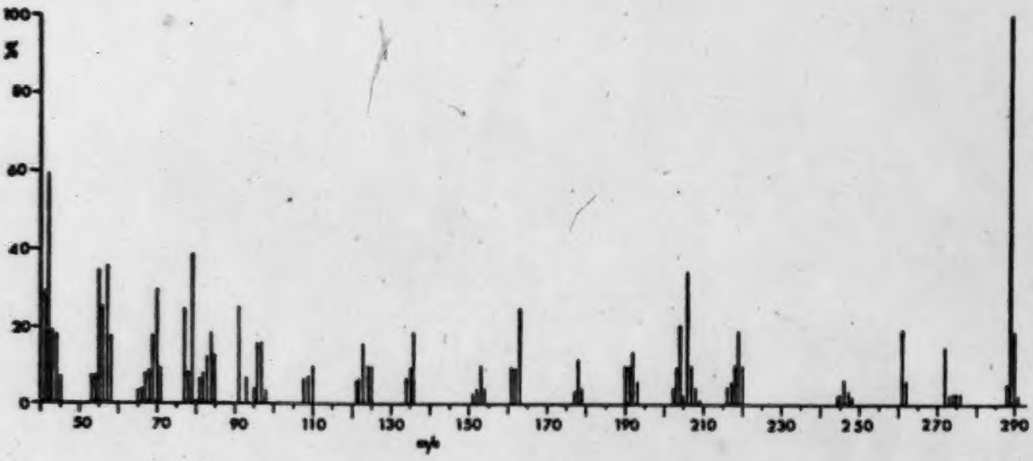


Fig. 13. The mass spectrum of the product of the oxidation reaction of lycoflexine.

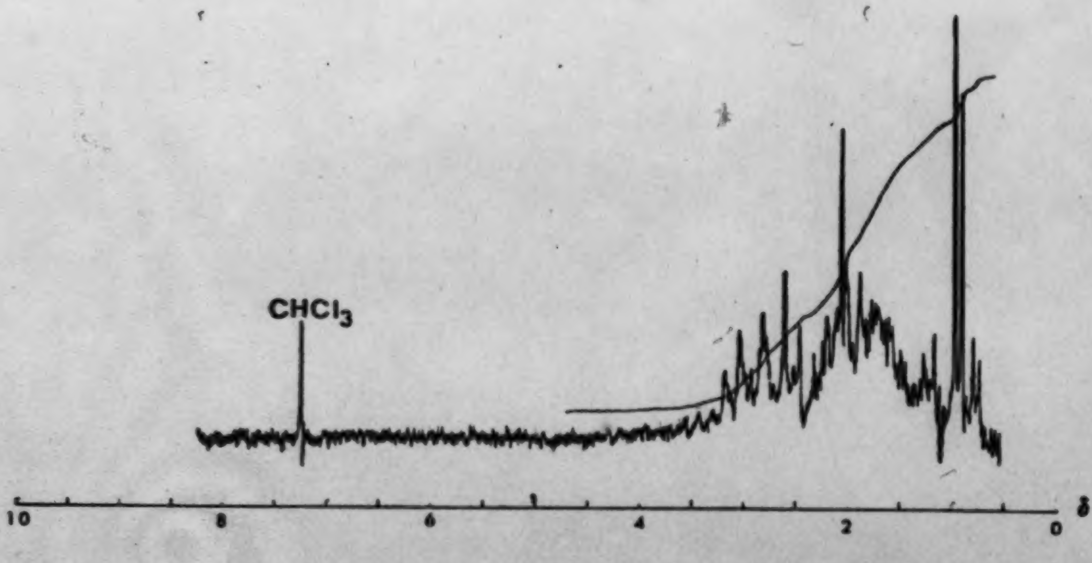
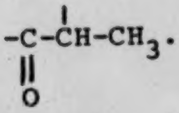
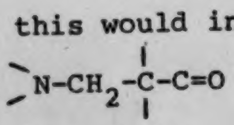
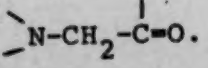
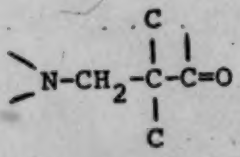
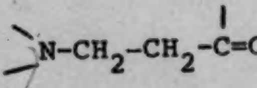
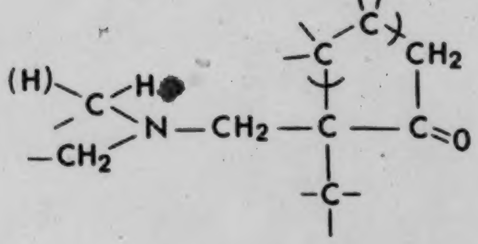


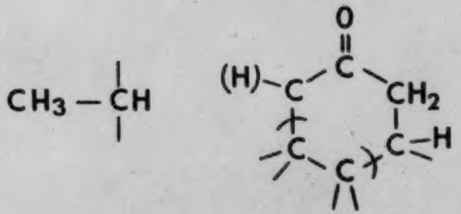
Fig. 14. The p. m. r. spectrum of deuterolycoflexine.

Examination of the p.m.r. spectrum (Fig. 14) of this compound gave some additional information. The methyl signal was still a clear doublet, eliminating the possibility of  $-\underset{\underset{O}{\|}}{C}-\overset{|}{C}H-CH_3$. More useful was the fact that the integration of the peaks corresponding to the hydrogens α to the nitrogen was in the same ratio as that corresponding to the methyl group in both lycoflexine and deuterolycoflexine. Of the two possibilities suggested earlier this would indicate the presence of the partial structure  $\rangle N-CH_2-\overset{|}{\underset{|}{C}}-C{=}O$ rather than  $\rangle N-CH_2-\overset{|}{C}{=}O$.

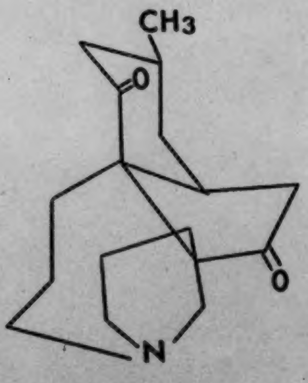
In fact, the low field regions are practically identical in the two spectra which would tend to indicate  $\rangle N-CH_2-\overset{\overset{C}{|}}{\underset{\underset{C}{|}}{C}}-\overset{|}{C}{=}O$ rather than  $\rangle N-CH_2-CH_2-\overset{|}{C}{=}O$, since deuteration would remove any vicinal H-C-C-H couplings and considerably simplify the spectrum.

The information so far indicates that lycoflexine contains the partial structures:

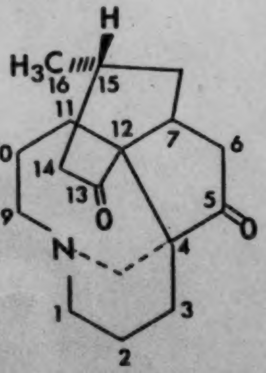




At this stage very little material remained. Since lycoflexine appeared to represent a new structural type an X-ray crystallographic study was undertaken by Dr. Y. Fukazawa in these laboratories. This was done on the hydrobromide salt, m.p. 266-267 (dec.). The structure of lycoflexine was shown to be as in (13a) and (13b) by this method.



(13a)



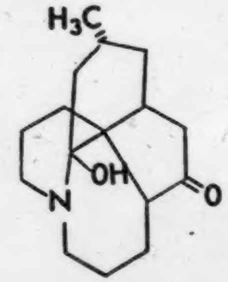
(13b)

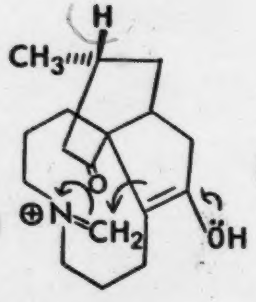
The structure of lycoflexine is similar to that of fawcettimine (12), with the addition of a methylene bridge between C-4 and the nitrogen. In fact condensation of fawcettimine with formaldehyde produced lycoflexine, presumably via an intermediate immonium ion (14). Since the absolute stereochemistry of fawcettimine is known[13] this conversion confirms the absolute stereochemistry of lycoflexine. The absolute stereochemistry established from the c.d. and o.r.d. spectra verifies the result.

The possibility that lycoflexine is an artifact (formed during the isolation process from fawcettimine and formaldehyde present in the technical grade methanol used) was eliminated by extracting the plant material directly with 5% aqueous hydrochloric acid and also separately with ether. Lycoflexine was found to be present in both cases and thus appears to be a true alkaloid.

Formation of lycoflexine in the plant probably occurs by a one carbon addition to fawcettimine, either by addition of a formaldehyde-type equivalent or oxidation of the corresponding N-methyl compound, similar to the oxidation encountered in the formation of the "berberine bridge"[14]. Formation of the bridge gives lycoflexine.

(12)





(14)

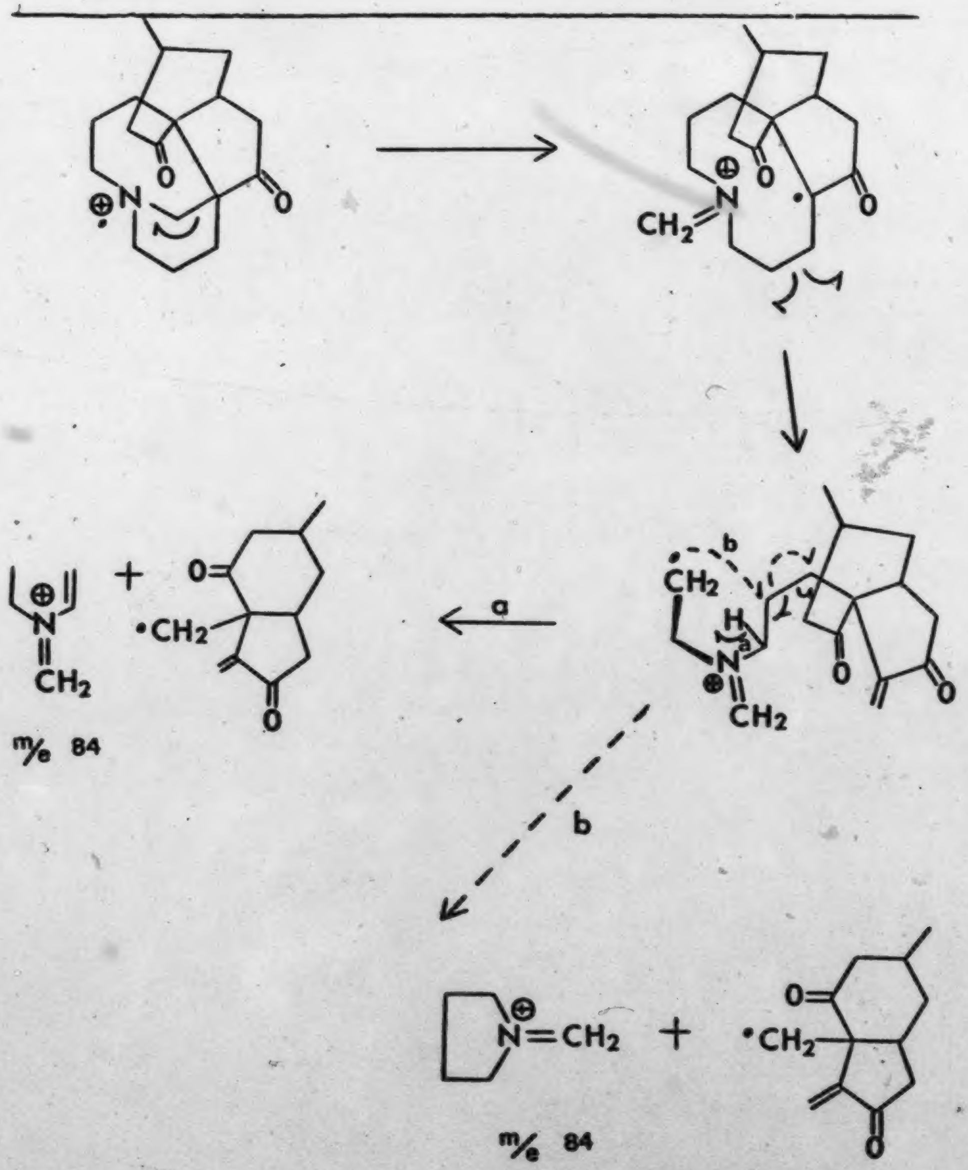
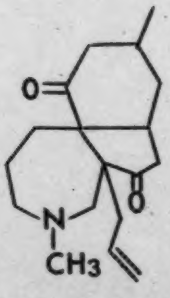


Fig. 15. A fragmentation scheme of lycoflexine.

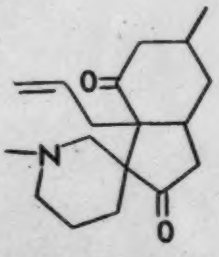
Even with the structure known, the mass spectral fragmentation to an m/e 84 ion is still not clear. A possible scheme is shown in Fig. 15. A second scheme avoiding an intermediate primary radical is shown in Fig. 16.

The rest of the data is consistent with the structure except for the deuteration reaction. The exchange of the fifth deuterium is not readily explicable, without invoking bond cleavage and subsequent bond reformation.

In the Hofmann elimination of lycoflexine methiodide two protons fill the *anti*-periplanar condition required for ease of elimination, those on C-2 and C-10. Elimination of these produces compounds (15) and (16) respectively. The formation of a simple Hofmann product is consistent with the mass spectrum, particularly the M-41



(15)



(16)

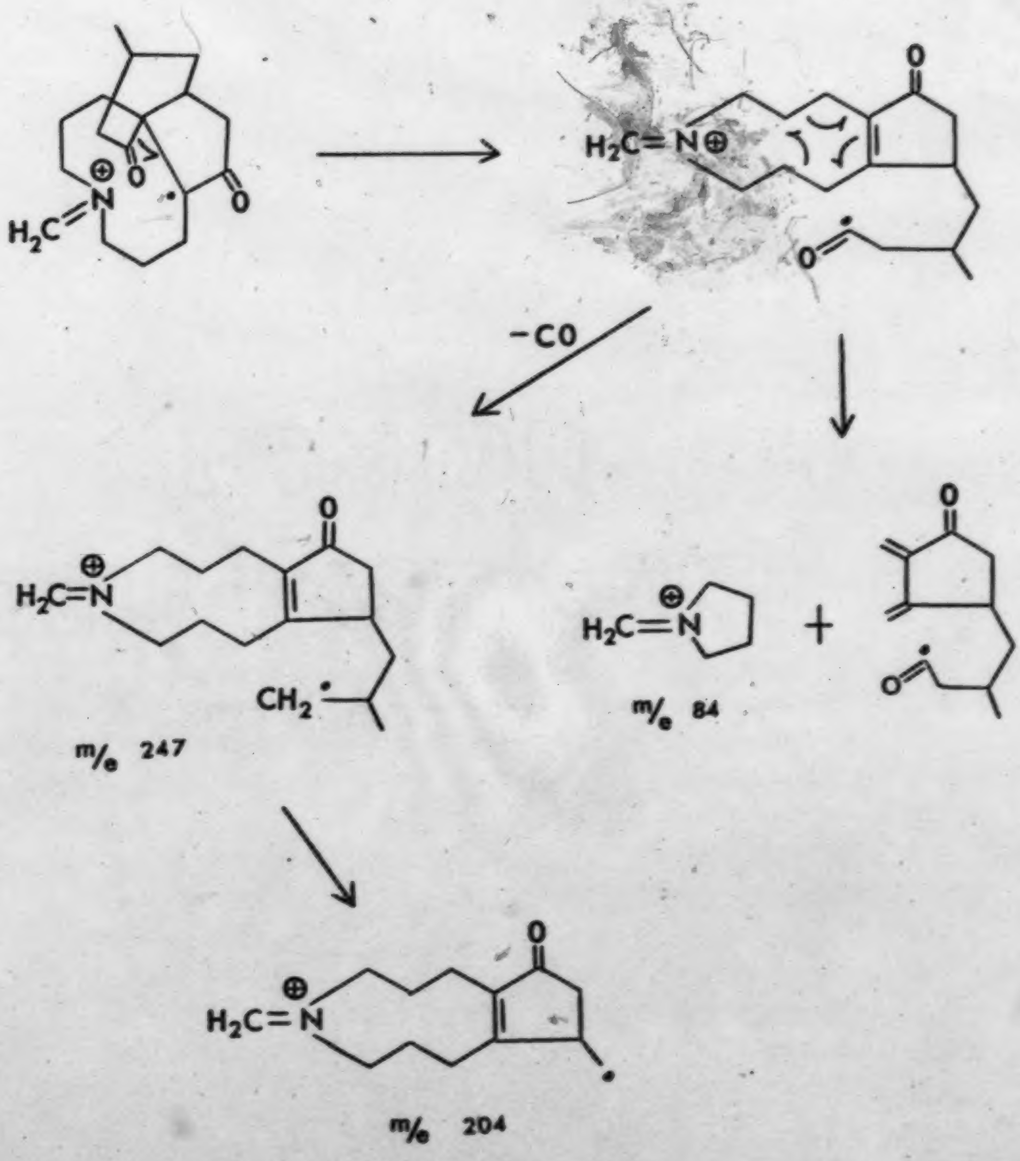
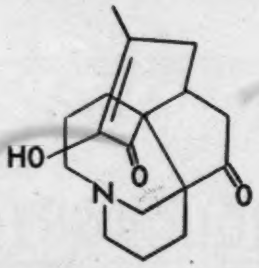


Fig. 16. Alternate fragmentation scheme of lycoflexine.

peak corresponding to loss of $CH_2{=}CH{-}\dot{C}H_2$. Possible fragmentation is shown in Fig. 17. However the distinction between (15) and (16) cannot be made on the data available.

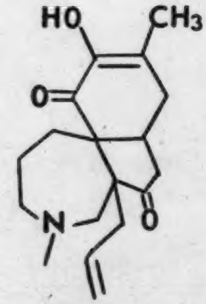
The i.r. and u.v. spectra of the oxidation product of lycoflexine indicated that the carbonyl in the six-membered ring becomes conjugated and suggests the probable structure (17) for this compound.



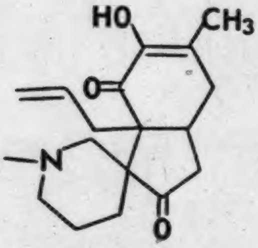
(17)

Thus the product from the reaction of both Hofmann elimination and oxidation has structure (18) or (19). This again is consistent with the major M-41 peak in the mass spectrum.

In agreement with the early supposition, lycoflexine represents a new skeletal type, the tenth encountered among the *Lycopodium* alkaloids.



(18)



(19)

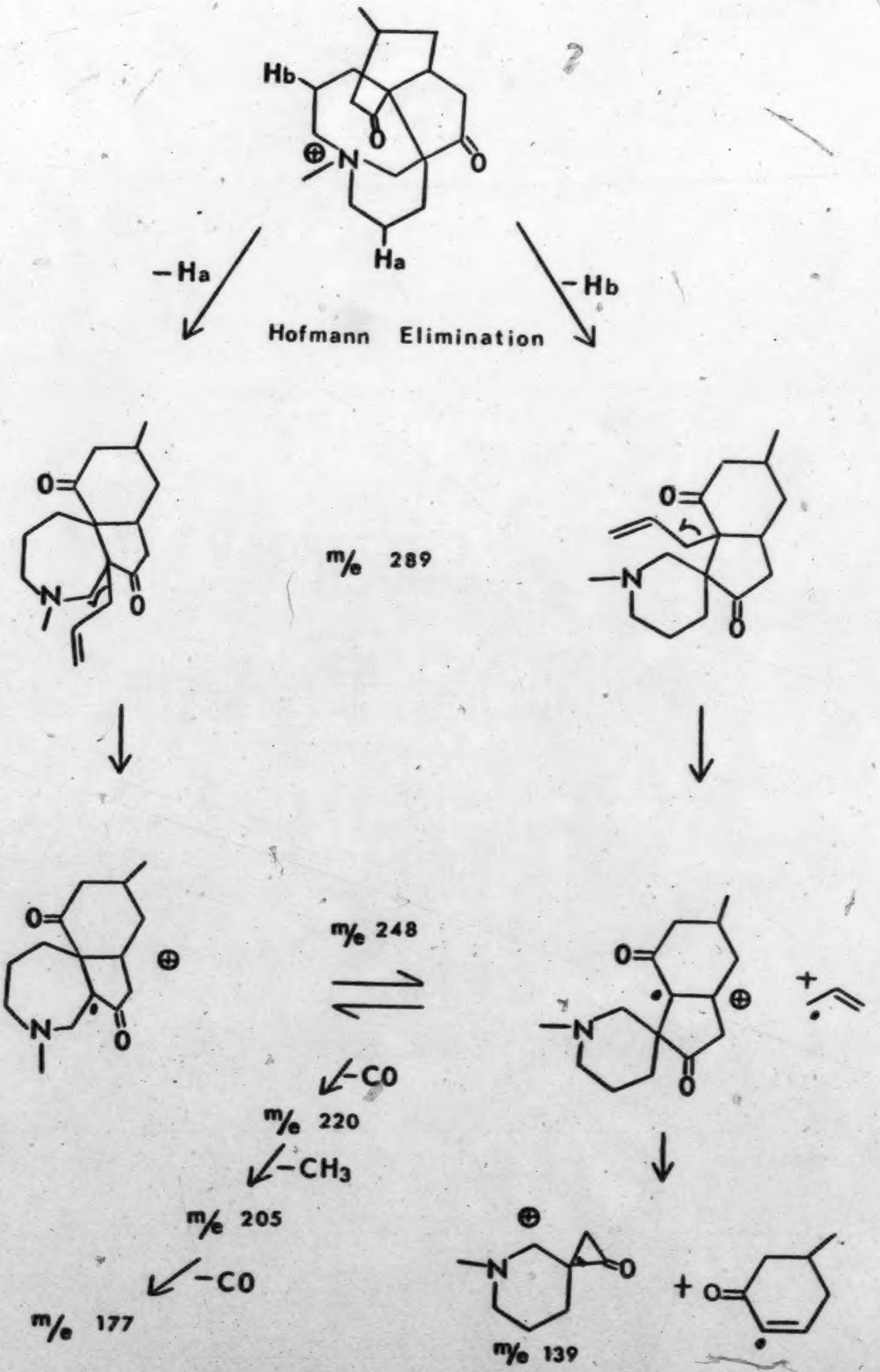


Fig. 17. A fragmentation scheme for the product of the Hofmann elimination reaction of lycoflexine methiodide.

[B] Hydroxylycopodine lactam

During the work on the isolation of lycoflexine from the *L. clavatum* var. *inflexum* alkaloids several other alkaloids were isolated. The major components are lycopodine and dihydrolycopodine; the minor, lycodoline, fawcettimine and a new alkaloid, $C_{16}H_{23}NO_2$, m.p. 200-202.5°.

Its mass spectrum (Fig. 18) shows peaks at m/e 277, 220 and 192 which correspond to $C_{16}H_{23}NO_3$, $C_{12}H_{14}NO_3$ and $C_{11}H_{14}NO_2$ as shown by high resolution mass spectroscopy. The molecular formula and m.p. showed it to be a hitherto unknown alkaloid but the fragmentation pattern, *i.e.*, loss of 57, is characteristic of a lycopodine-type skeleton[9], as discussed earlier, and immediately suggests this carbon-nitrogen skeleton.

Its i.r. spectrum shows absorption at 3400, 1720 and 1630 cm^{-1} (Fig. 19). These three absorptions account for the three oxygen functions, *i.e.*, hydroxyl, ketone carbonyl and amide carbonyl.

The p.m.r. spectrum (Fig. 20) shows a doublet (J=6c.p.s.) at δ 0.8 corresponding to $C\underline{H}_3$-CH. A multiplet around δ 4.3 corresponds to protons on carbons adjacent to the nitrogen which overlap the signal of the carbinol

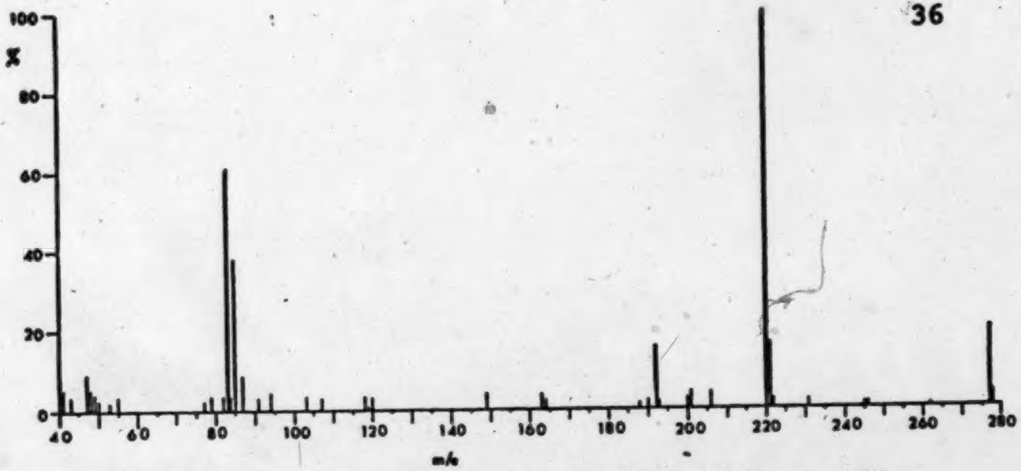


Fig. 18. The mass spectrum of hydroxylycopodine lactam.

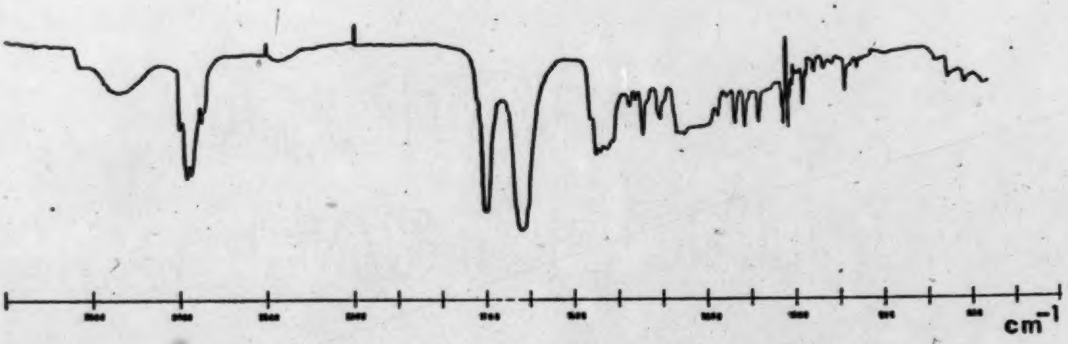


Fig. 19. The i. r. spectrum of hydroxylycopodine lactam.

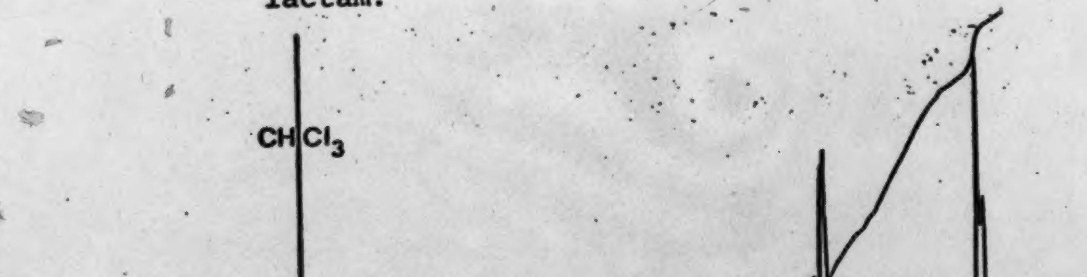
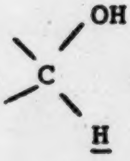
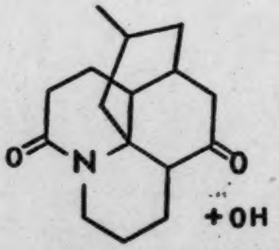


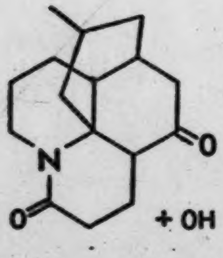
Fig. 20. The p. m. r. spectrum of hydroxylycopodine lactam.

proton, . There are no signals due to olefinic protons.

The u.v. spectrum shows that there is no conjugated system present. This initial spectral analysis suggested the compound is:

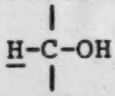
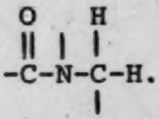
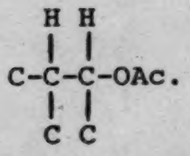


or



The compound was acetylated with acetic anhydride and pyridine at room temperature and thin layer chromatography indicated the formation of a derivative. The mass spectrum of this monoacetate has peaks at m/e 319; 259; 262; 202 and 174 corresponding to the loss of acetic acid, (60 mass units), the bridging carbons, (57 mass units) and CO (28 mass units).

The formation of a monoacetate is confirmed by the i.r. spectrum (Fig. 21) which shows a new carbonyl band at 1730 cm^{-1} and the corresponding loss of hydroxyl absorption.

The p.m.r. spectrum of the monoacetate (Fig. 22) provides further information. The signal originally due to  $\underline{H}-\overset{|}{\underset{|}{C}}-OH$ shifts downfield on acetylation[15] to δ 5.39. A doublet of doublets (1H) at δ 4.50 is now clearly visible and is coupled to a triplet (1H) at δ 2.96 (J=14c.p.s.). These signals which are further coupled, are due to the protons  $-\overset{\overset{O}{\|}}{C}-\overset{|}{N}-\overset{\overset{H}{|}}{\underset{|}{C}}-H$. The proton geminal to the acetoxyl was shown to be coupled to a proton resonating at δ 1.64 (J=4c.p.s.). Decoupling the δ 1.64 signal produces a sharp singlet for the δ 5.39 signal indicating the partial structure  $C-\overset{\overset{H}{|}}{\underset{\underset{C}{|}}{C}}-\overset{\overset{H}{|}}{\underset{\underset{C}{|}}{C}}-OAc$. This would suggest C-6 or C-14 for the hydroxyl group as these are the only positions having only one hydrogen on the adjacent carbon. However C-14 is eliminated since it is on the "bridge" (which is unsubstituted as shown by the mass spectrum), hence the probable structure is:

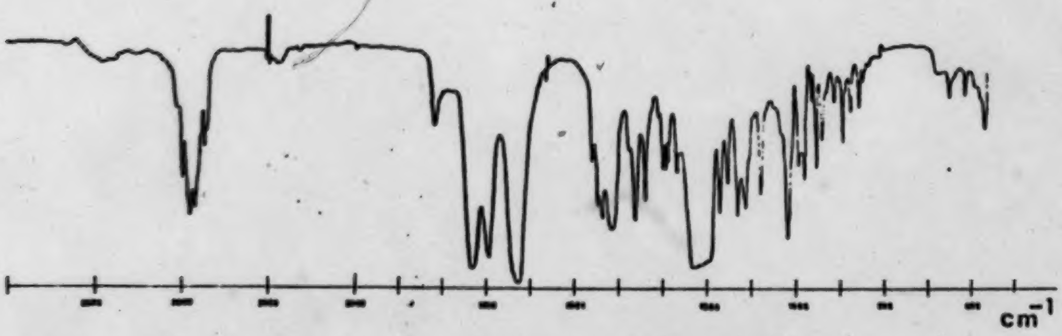


Fig. 21. The i. r. spectrum of lycopodine lactam monoacetate.

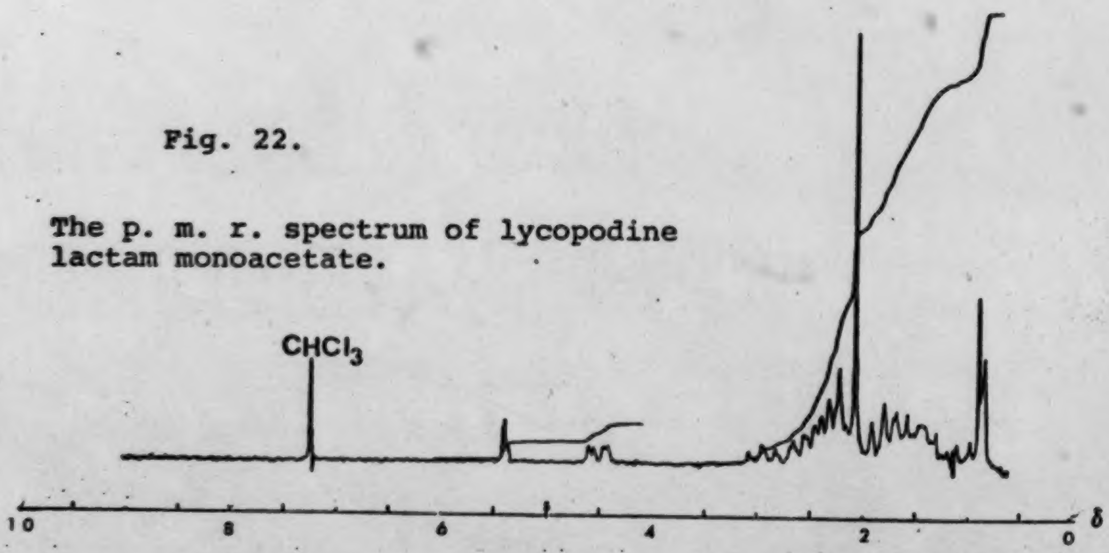
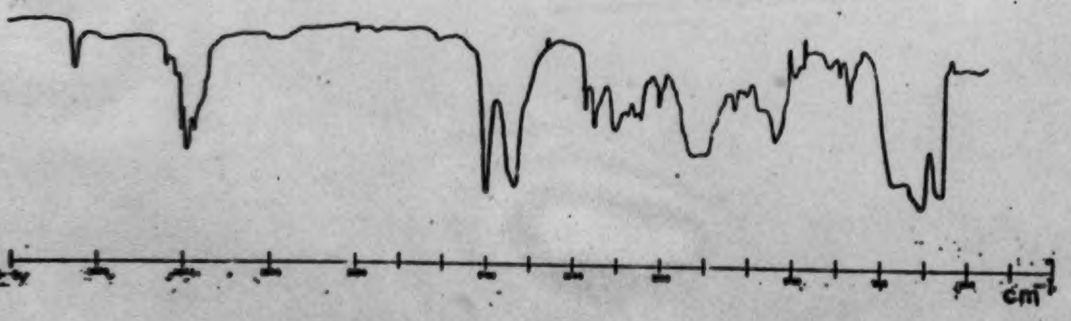
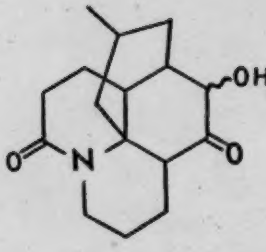


Fig. 22.

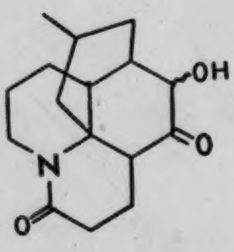
The p. m. r. spectrum of lycopodine lactam monoacetate.

Fig. 23. The i. r. spectrum of chlorolycopodine lactam.



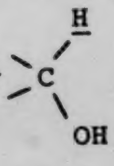


or



If the alkaloid or its monoacetate is heated under reflux in acetic anhydride with pyridine as catalyst, the diacetate is formed. The mass spectrum of this compound displays peaks at m/e 361 ($C_{20}H_{27}NO_5$); 319 ($C_{18}H_{25}NO_4$); 304 ($C_{16}H_{18}NO_5$); 301 ($C_{18}H_{23}NO_3$); 262 ($C_{14}H_{16}NO_4$); 259 ($C_{16}H_{21}NO_2$); 220; 202 ($C_{12}H_{12}NO_2$) indicating loss of acetic acid and the bridge but no loss of CO, thus suggesting the formation of an enol acetate.

In order to locate the position of the hydroxyl function an attempt was made to dehydrate the alkaloid. Treatment with thionyl chloride in methylene chloride produced a new compound, m.p. 102-105°.

The i.r. spectrum (Fig. 23) shows the loss of the hydroxyl group and the retention of the amide and keto functionality. There was however no indication of the presence of an olefinic bond. The u.v. spectrum indicates that a conjugated system was not present. The p.m.r. spectrum was little changed from the starting compound except that the signal at δ 4.5 for  was no longer apparent. The mass spectrum with peaks at m/e 295 ($C_{16}H_{22}NO_2{}^{35}Cl$); 260; and 238 ($C_{12}H_{13}NO_2{}^{35}Cl$) shows that the hydroxyl group has been replaced by chlorine.

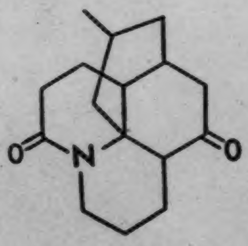
Attempts to eliminate hydrogen chloride, either with pyridine[16] or with sodium methoxide[17] in methanol failed, in both cases the starting chloro compound was recovered unchanged.

As an alternate method of dehydration an attempt was made to form the mesylate by treatment with methanesulfonyl chloride and pyridine[18]. Again the product from this reaction was the chloro compound.

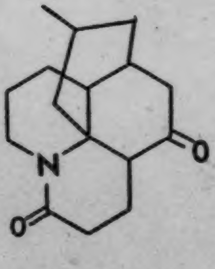
The apparent ease with which the hydroxyl group was replaced and the difficulty in eliminating the chlorine is unusual for a simple system. The coupling constants in the p.m.r. spectrum suggest the hydroxyl group is axial, and the difficulty of the elimination reaction makes C-6 seem the most likely position of substitution since elimination would lead to an *anti*-Bredt double bond.

Treatment of the chloro compound with zinc and glacial acetic acid gave the dechloro compound[19]. The mass spectrum of this compound shows peaks at m/e 275 ($C_{16}H_{21}NO_3$); 247 ($C_{15}H_{21}NO_2$) and 220 ($C_{12}H_{14}NO_3$).

Hence zinc will reduce the chloro compound to a lycopodine lactam. There are two possible lactams (20)[20] and (21)[21]. These have identical R_f values but different i.r. spectra, different mass spectra, and different reported m.p., 163° and 177° respectively.

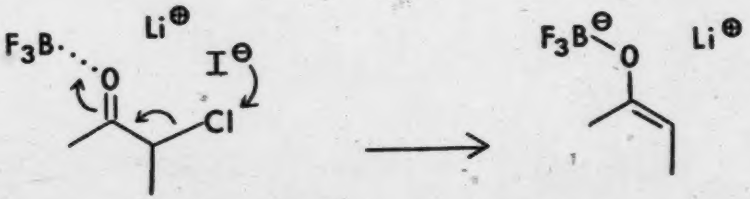


(20)

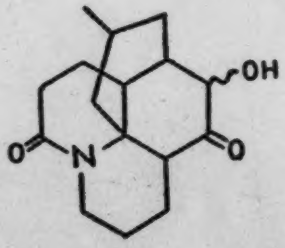


(21)

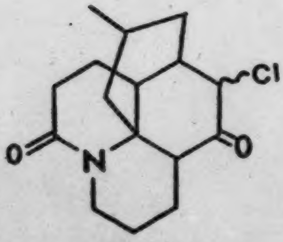
The chloro compound was stirred with lithium iodide and boron trifluoride etherate in ether[22]. After purification by chromatography over alumina, the reaction product was shown to have identical i.r. and mass spectra with lycopodine lactam (20). This reaction is specific for α-halo ketones since it involves the reaction mechanism.



Hence the alkaloid is hydroxylycopodine lactam (22) and the chloro compound is (23).

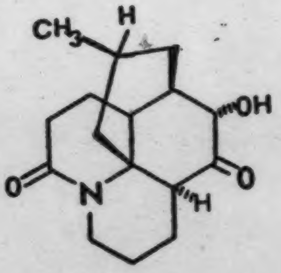


(22)



(23)

No attempt was made to rigourously prove the stereochemistry of the hydroxyl group. However the stretching frequency of the keto function is unchanged in the i.r. spectrum of the chloro compound. This suggests it is in the axial position as there is a substantial field effect from an equatorial halogen[23]. Normally reactions with thionyl chloride do not involve inversion of configuration[24] and thus the hydroxyl group is also axial. This is also consistent with the n.m.r. data. Therefore the compound is 6-α-hydroxylycopodine lactam (24). This is the first reported occurrence of a lactam alkaloid among the Lycopodium alkaloids.

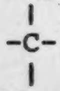
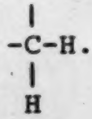
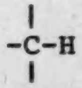


(24)

[C] Carbon 13 n.m.r. of some *Lycopodium* Alkaloids

During the course of the work on lycoflexine carbon 13 n.m.r. (c.m.r.) spectroscopy was considered as a possible aid in the structure elucidation. Since at that time no c.m.r. work had been done on the *Lycopodium* alkaloids, we decided to determine the spectra of the more common alkaloids of the group to investigate the potential of the method. As it developed, the structure of lycoflexine was solved before instrumentation became available to determine c.m.r. spectra on the small quantities available. However, the investigation of the more common alkaloids proved to be an interesting problem in itself and is described below.

The instrumentation for c.m.r. spectroscopy has advanced greatly in the last five years and spectra can now be readily obtained using half millimole or smaller samples, consequently it is becoming increasingly important in organic chemistry. Thus far no general theoretical treatment which allows accurate prediction of chemical shifts has been advanced. Consequently when using c.m.r. as a tool for structure elucidation the assignment of chemical shifts relies heavily on comparison with model compounds where the shifts have been unambiguously assigned[25,26].

The shift ranges for different types of carbon functions are shown in Fig. 24[25,26]. Specific assignments are made using a variety of techniques. An important method is that of "off resonance decoupling"[27], irradiation of the protons at frequencies differing slightly from their resonance frequency which results in a residual coupling between the C and H nuclei. Methyl carbons appear as quartets, methylene carbons as triplets, and so on. In practice the partially decoupled spectrum is laid over the completely decoupled spectrum and if there is a coincident peak in both spectra the signal is from  $-\overset{|}{\underset{|}{C}}-$ or  $-\overset{|}{\underset{\underset{H}{|}}{C}}-H$. If there is no coincident peak it is from  $-\overset{|}{\underset{|}{C}}-H$ or $-CH_3$. Quarternary carbons can be identified from the undecoupled spectra since they are the only unsplit signals.

When necessary it is possible to selectively decouple a single set of equivalent protons, provided they are separated from other ^1H resonances in the p.m.r. spectrum, and so identify a specific carbon nucleus[28]. This is particularly useful for methyl groups.

The peak area is not generally useful. With symmetrical molecules where two carbons are equivalent the signal will usually be twice the intensity as that from non-equivalent carbons. However, if a nucleus cannot relax (normally by dipole-dipole interaction with adjacent hydrogen nuclei), it will "saturate" and produce a signal of reduced intensity. Variable nuclear Overhauser effects[26] can also lead to variations in peak height. Sometimes measurement of the relaxation time, T_1, or a reduced peak height will help in the assignment of a nucleus[29].

Another major method of identifying a signal is by introduction of deuterium into the molecule[30]. This effectively "washes out" the signal of the carbon to which the deuterium is attached, and often causes a slight shift in the signal of the adjacent carbon as well.

For nitrogen containing compounds protonation of the nitrogen produces a shift in most of the resonances of the molecule[31]. This effect has been examined and is greater the closer a nucleus is to the site of protonation. Experimentally, the shift is measured as the acid concentration is increased enabling signals from the acidified sample to be compared with the signal from the same carbon in the non-acidified sample.

Finally, using known compounds it is possible to formulate a table of "additivity parameters" as in Fig. 25[32]. Thus it is possible to calculate the change in shift produced by a substituent in a nearby position. Steric effects are also very important and must be taken into account[33].

Lycopodine-type alkaloids present an interesting problem since they are relatively large, cage-type molecules, having two or more heteroatoms. These factors combine to produce electronic and steric interactions which complicate the assignment of the chemical shifts. All of the above procedures were used to assign the resonances in the c.m.r. spectrum of lycopodine.

It was decided to proceed with a series of compounds: lycopodine (1), dihydrolycopodine (25), epidihydrolycopodine (26), flabelliformine (27), clavolonine (28), lycodoline or "L-8" (29), epilycodoline or "L-23" (30), α-lofoline (31), and lycodine (3), (Chart 2) to relate the spectral changes to structural changes and to assign the chemical shift of each carbon nucleus in each compound.

The first eight compounds all have the basic lycopodine skeleton while lycodine has a different skeleton including an aromatic ring not present in the others.

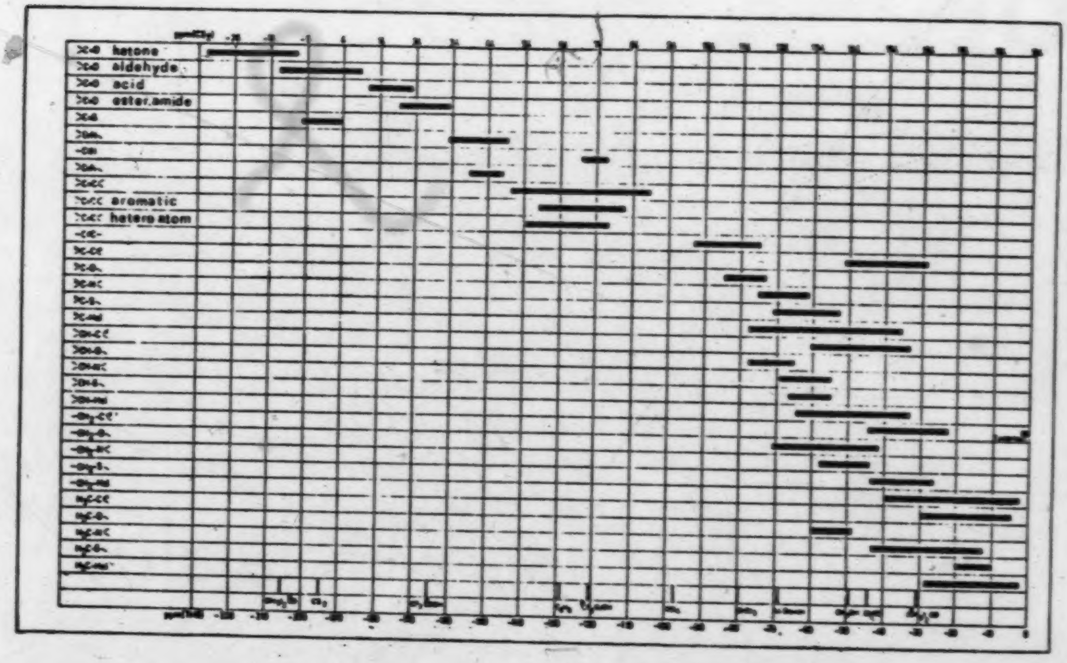
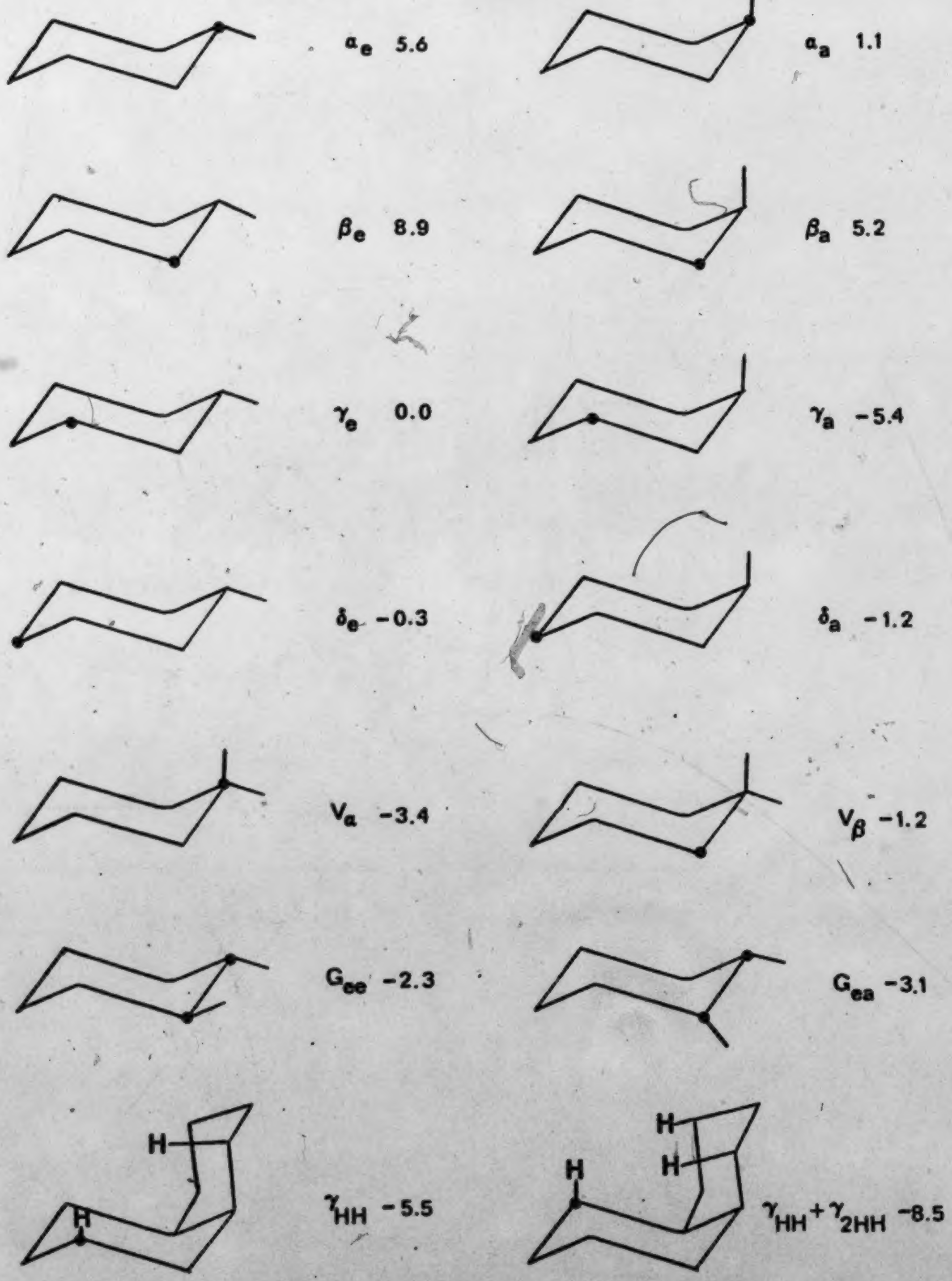
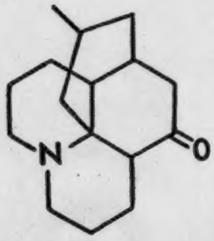


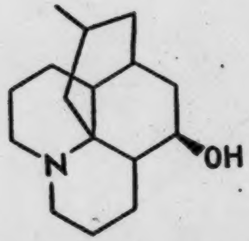
Fig. 24. The chemical shift of various types of carbon nuclei.

Fig. 25. The additivity parameters for substituted cyclohexanes.

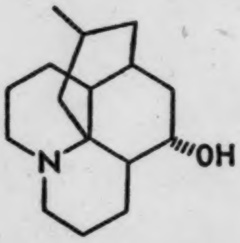




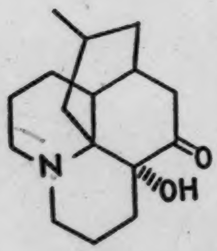
Lycopodine (1)



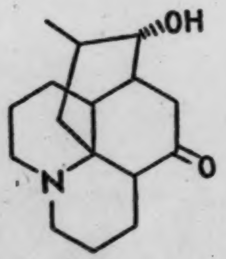
Dihydrolycopodine(25)



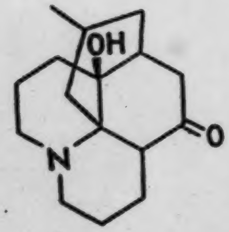
Epidihydrolycopodine(26)



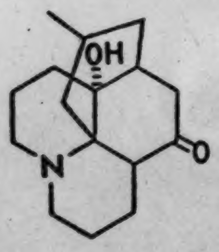
Flabelliformine(27)



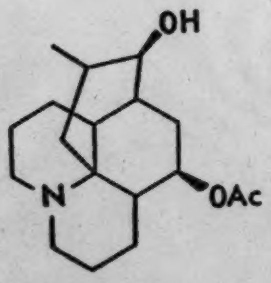
Clavolonine(28)



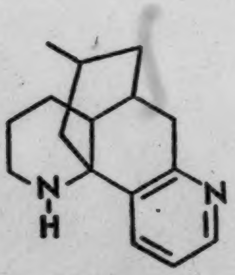
Lycodoline(29)



Epilycodoline (30)



α - Lofoline(31)



Lycodine (3)

CHART 2. Alkaloids of the lycopodine structural type.

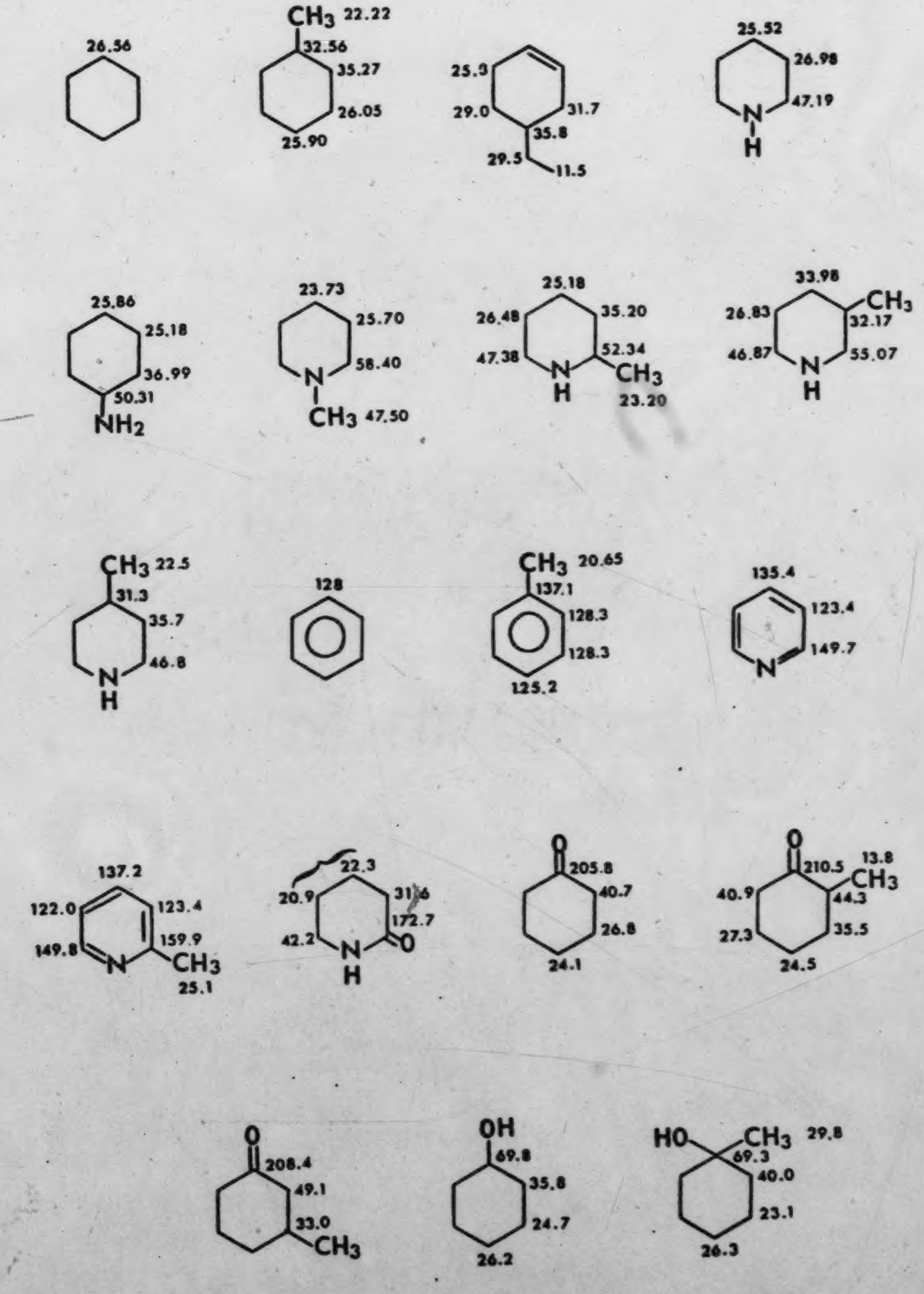
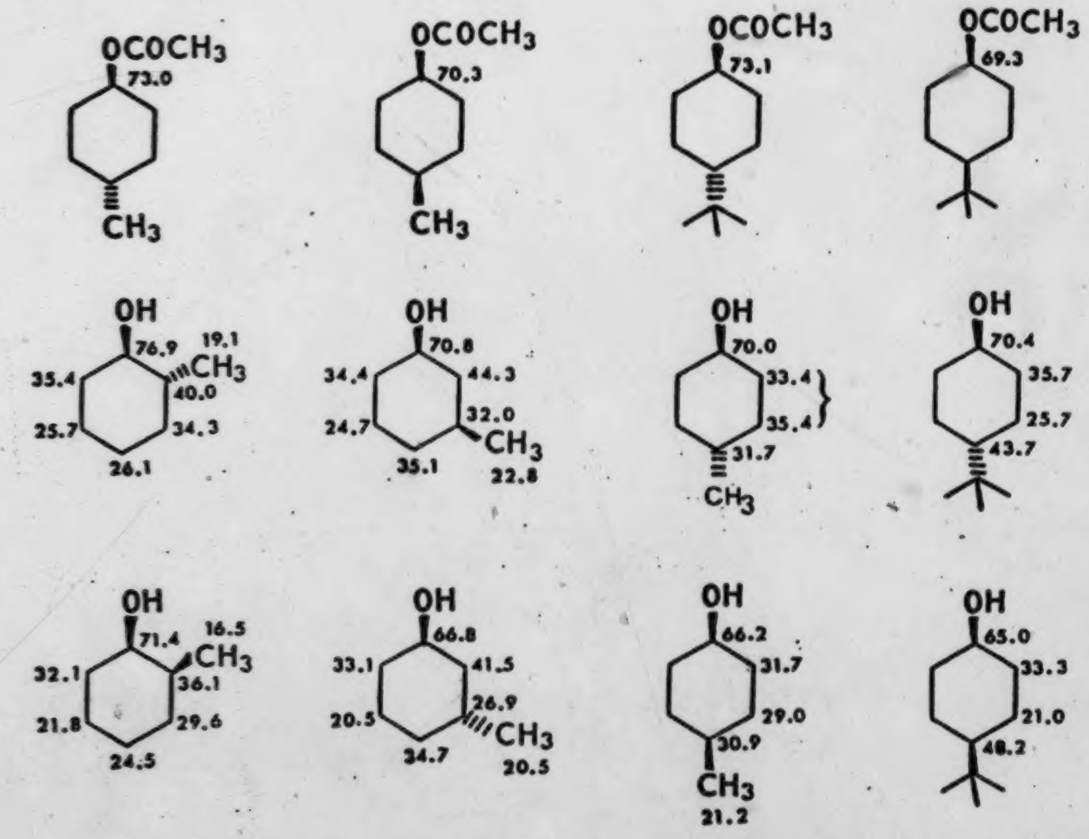


CHART 3. Chemical shifts of carbon nuclei of selected model compounds.

CHART 3 (con't)



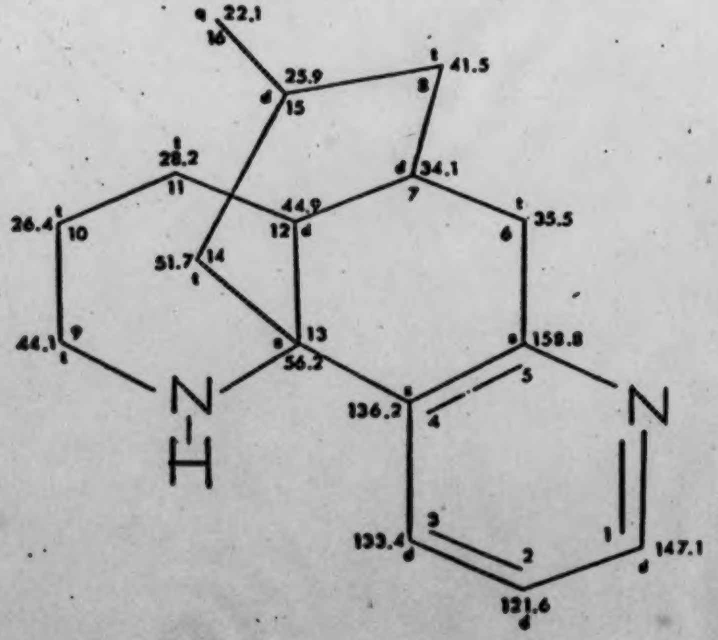


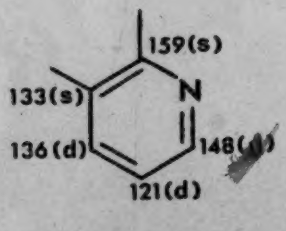
Fig. 26.

The c.m.r. data of lycodine.

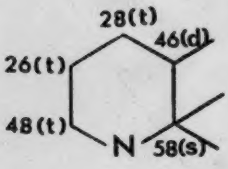
Lycodine has a less complicated c.m.r. spectrum than the others, and once the resonances had been assigned, it served as a useful model for some positions in the lycopodine-type alkaloids.

The c.m.r. spectral data of lycodine is listed in Table 1 (page 78) while Fig. 26 shows the shift assignment and the multiplicity of the signals. The assignments rely heavily on a comparison with the known δ values for α-picoline[34], pyridine[35], methylpiperidines[36], methylcyclohexanes[37] and cyclohexylamine[38] (Chart 3).

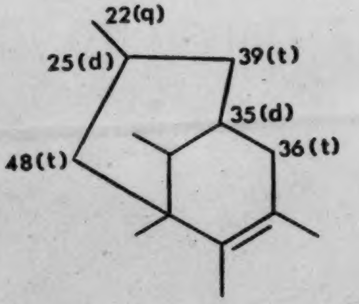
The five low field signals between 120 and 160 p.p.m. are assigned to the aromatic carbons. Useful models for this system are α-picoline and pyridine, which can be compared to benzene and toluene (Chart 3) for the effect of substituents[39]. The expected chemical shifts and multiplicities are:



Using the methylpiperidines as models and the additivity parameters, the expected shifts in the A ring are:



Methylcyclohexane and cyclohexylamine are the models for the carbocyclic ring and allowing for axial interactions where present, the expected resonances are:



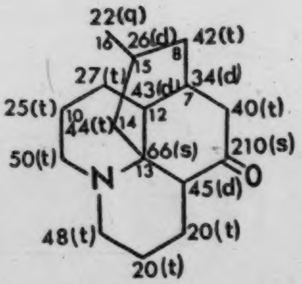
The expected position for C-6 is calculated using decalin[40] and 4-ethyl-cyclohexene[41] (Chart 3) as models and making correction for the expected effect of the nitrogen.

For this simple system there is good agreement between the expected results calculated from model compounds and additivity parameters, and the experimental results. The assignment of shifts follows readily as shown in Fig. 26.

The compounds in the lycopodine series have steric interactions of the "γ_{HH}" and "γ_{2HH}" types (the nomenclature is that of Dalling and Grant) as in Fig. 25, not present in lycodine. However the magnitude of these interactions has been determined for decalins[33] and perhydroanthracenes[42] and should be similar in the lycopodine system.

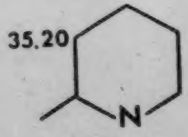
The c.m.r. resonances for each alkaloid have been assigned as far as possible and are shown in Table I. A few assignments are less certain than others and these are indicated. The methods by which these assignments were made is discussed below, particularly for the case of lycopodine, Fig. 27.

The models for lycopodine are N-methyl, 2-methyl, and 3-methylpiperidine[36], cyclohexylamine, methylcyclohexane, the methylcyclohexanones[43] (Chart 3) and lycodine. The effect of α, β and γ substituents are calculated using the values in Fig. 25. Carbons 1, 2, 3, 4, 6, 9, 11, 14 experience a γ_{HH} interaction and of these carbons 4, 9, 11 and 14 experience an additional γ_{2HH} interaction. The resonances expected for lycopodine are:



The positions numbered are in similar environments in lycodine and lycopodine hence predictions are expected to be closer to the experimental values for these positions. An example of how these values are calculated is shown for position 4: -

Using as the closest model:



there is a β_{ax} substituent (+5.4 p.p.m.), a carbonyl in the α position (+11.8 → +14.1 p.p.m.) and a $\gamma_{HH} + \gamma_{2HH}$ interaction (-8.5). Therefore the expected value is 35.2 + 5.4 + 11.8 (or 14.1) - 8.5 = 43.9 → 46.2. The smaller value for the effect of the carbonyl is obtained by comparing 2-methylcyclohexanone with methylcyclohexane, while the greater value is from comparison of cyclohexanone with cyclohexane. Thus the expected resonance position of C-4 is about 45 p.p.m.

The assignment of the experimentally determined values is shown in Fig. 27. The signal at 213.4 p.p.m. is assigned to the C-5 carbonyl carbon. The signal at 59.9 p.p.m. (s) is assigned to the highly substituted C-13 and signals at 47.2 p.p.m. (t) and 47.8 p.p.m. (t) to the other carbons α to nitrogen (C-1 and C-9). On deuteration of the C-9 position, by oxidation to the lactam and reduction with lithium aluminum deuteride and finally oxidation back to lycopodine, the 47.8 p.p.m. signal disappears, confirming the assignment of C-9 and thus the 47.2 p.p.m. signal must originate from C-1.

Deuteration of the C-4 and C-6 positions removes a doublet at 43.4 p.p.m., assigned to C-4, and a triplet at 43.3 p.p.m. assigned to C-6. This enables the distinction

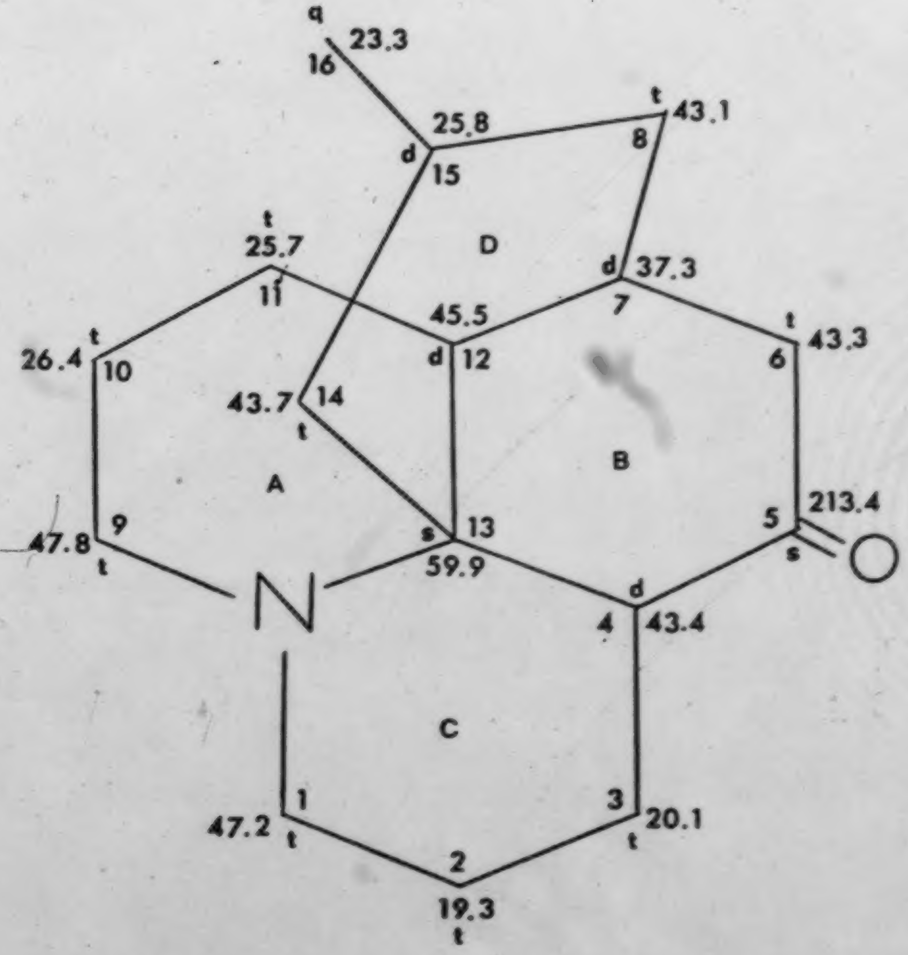
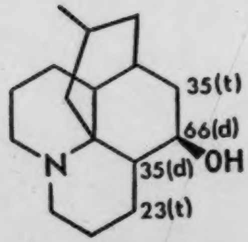


Fig. 27. The c. m. r. data of lycopodine.

to be made between C-4 and C-12, the latter being assigned the shift at 45.5 p.p.m. The two remaining doublets at 37.3 p.p.m. and 25.8 p.p.m. may now be assigned with confidence to C-7 and C-15 respectively. The 23.3 p.p.m. signal should be that of the C-16 methyl carbon, which leaves two signals occurring at higher field which at first seems unusual. However selective decoupling of the methyl protons confirmed this assignment. Three pairs of signals remain, those of C-8, C-14, C-10, C-11 and C-2, C-3. C-8 is expected to resonate at slightly higher field than C-14. The assignment was verified by observing the change in chemical shift on acidification. Protonation of the nitrogen should affect C-14 (β to N) much more than C-8 (δ to N); the 43.7 p.p.m. signal shifts -4.5 p.p.m. and is assigned to C-14, whereas the 43.1 p.p.m. signals shifts only -1.6 p.p.m. and is assigned to C-8. Similarly the 26.4 p.p.m. signal shifts -3.4 p.p.m. and is assigned to C-10 (β to N) whereas the 25.7 p.p.m. signal shifts only -2.1 p.p.m. and is assigned to C-11 (γ to N). The change in shift for C-10 and C-11 is much less than for the previous pair and it is possible that the C-10 and C-11 assignments should be reversed. The C-2, C-3 assignments are less certain. On deuteration of C-4 there is a small shift (about 0.05 p.p.m.) of the 20.1 p.p.m. signal and hence this is assigned to C-3 and by exclusion the 19.3 p.p.m. signal to C-2[44]. Conflicting results arise from the acidification experiment. The change in the C-2 (β to N) shift is expected to be greater than that of the C-3 (γ to N) shift, however the opposite was in fact found to occur. Possibly the proximity of the carbonyl group is responsible for this anomalous behavior ~~or~~ perhaps the assignment should be reversed. The complete results of the acidification experiment are shown in Table 2. It is interesting to note the shifts for C-4 and C-13 on acidification. These are deshielded while all the other nuclei are shielded. This is likely due to the higher substitution at these positions as later results preclude any lone pair - π interaction in the ground state[45]. With the assignments of the carbons in lycopodine established it is now considerably easier to assign the resonances in the rest of the series. When considering the other members of the series changes mentioned are with respect to lycopodine unless otherwise specified.

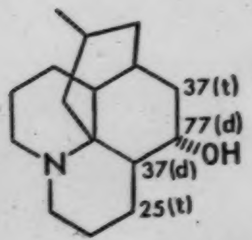
The next in the series is dihydrolycopodine (25). The substitution of the hydroxyl function for the carbonyl should change several resonances as shown below. Positions not marked are the same as in lycopodine. The new resonances expected are:



Models for this system, in addition to those previously used, are the methylcyclohexanols[46] (Chart 3) and of course lycopodine. The effect of the axial hydroxyl function on the adjacent positions, compared to the keto function, is to shift them about 8 p.p.m. upfield, but, using cis-2-methylcyclohexanol as the model the C-3 shift is expected only to be about 3 p.p.m. upfield.

Assignments in agreement with the experimental results follow readily. C-9 is assigned at lower field than C-1 by analogy with lycopodine. The compound deuterated at the C-4 and C-6 positions was readily available from lycopodine-$\underline{d}_2$ and was used to confirm the assignment of these nuclei. The assignments of the C-10 and C-11 carbons might be reversed as before and there is also the possibility that the C-3 and C-11 assignments could be interchanged, however, the results are consistent with those obtained upon acidification.

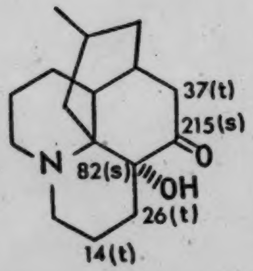
Epidihydrolycopodine (26) will be similar to dihydrolycopodine except the hydroxyl group is now equatorial and the adjacent positions are expected to have the resonances below:



The equatorial hydroxyl group shifts the adjacent carbon resonances about 6 p.p.m. upfield (compared to 8 p.p.m. for axial hydroxyl) and the β-carbon resonances 5 p.p.m. upfield (compared to 3 p.p.m. for axial hydroxyl). As before the remaining positions are relatively unchanged from lycopodine and resonance positions are easily assigned. The C-3, C-10 and C-11 positions might be interchanged as in the previous example.

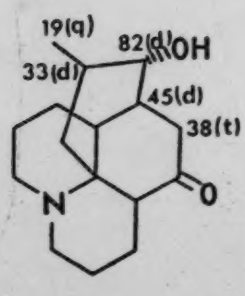
Comparing the results from dihydro- and epidihydrolycopodine brings out some interesting points. The axial hydroxyl group has a greater steric effect on C-15 than the carbonyl but the equatorial hydroxyl group produces less crowding, thus in the former C-15 resonates at 23.5 p.p.m. and in the latter at 28.9 p.p.m. compared to 25.8 p.p.m. in the case of lycopodine. The relative position of the C-4 and C-6 resonances is reversed in the two compounds. This is probably due to the fact that in dihydrolycopodine ring B tends to be flattened to reduce nonbonded interactions[47], bringing the H-4 closer to the H-9 thus increasing the steric crowding about the C-4 nucleus and producing greater shielding. The C-4 and C-13 shifts on acidification are the same or greater than in the case of lycopodine, even though no "σ coupled p" interaction is possible since there is no longer any π-bond.

The next compound in the series, flabelliformine (27) has an axial hydroxyl group in the 4 position. The hydroxyl group is expected to be directed out of the ring. Hence its steric effect will not be very large (although greater than H) and C-9 and C-11 should be more or less unchanged from lycopodine. C-2 and C-6 however are expected to be about 5 p.p.m. upfield because of the γ_a effect. Those resonances differing from lycopodine are shown below:



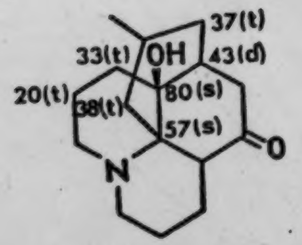
From this the assignments in Table 1 are easily made, allowing for the fact that C-3, C-10 and C-11 may be interchanged. The axial hydroxyl group does not seem to be causing as much steric crowding as expected, C-2 and C-6 resonating about 2-3 p.p.m. downfield from the expected value. This may indicate that the H-bonding between the hydroxyl and the carbonyl tends to distort the ring B geometry somewhat or that it simply reduces the effective steric size of the hydroxyl group. The results from the acidification experiment support these assignments.

Clavolonine (28) is 9-hydroxylycopodine with the hydroxyl group in an equatorial position. Once again those resonances differing from lycopodine are expected to be as below:



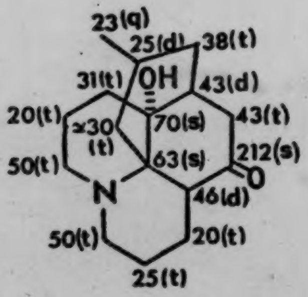
The expected values for C-16 and C-8 are obtained using *trans*-2-methylcyclohexanol as a model. The C-5 and C-7 resonances are expected to be about 8 p.p.m. downfield from the lycopodine values. The interaction between the O-*H* and H-6 is similar to the γ_{HH} interaction and so C-6 is expected to be shielded by about 5.5 p.p.m. With these predictions the assignments can be made as shown. Again there is the possibility that C-2 and C-3 as well as C-10 and C-11 may be interchanged. The results obtained on acidification again are consistent with the assignments.

Lycodoline (*29*) is 12-β-hydroxylycopodine and is slightly more complex as there is a nitrogen-hydroxyl hydrogen bond. This holds the hydroxyl group inside the ring and introduces steric crowding of C-8, C-10 and C-14 comparable to that caused by a methyl group and is expected to shield these positions by about 6 p.p.m. Positions changed from lycopodine are:



Positions 7, 11 and 13 should shift about 8 p.p.m. downfield. Using 1-methylcyclohexanol as the model, the C-12 resonance should be around 80 p.p.m. With these assumptions the assignments can be made as shown. It is seen that the steric effect of the hydroxyl group is greater than expected, shielding C-14 by 7.6 p.p.m., but the electronic effect is less, deshielding adjacent nuclei only by 2-5 p.p.m. This result is possibly due to the strong hydrogen bonding of the hydroxyl group to the nitrogen. Furthermore the carbinol carbon is shielded more than expected, reflecting strong steric interactions again possibly due to the strong hydrogen bond. There is once more the possibility that the C-2 and C-3 or C-3 and C-10 shifts may be interchanged.

The epimer of lycodoline, "L-23" (30), is an even more complex case. Again there is a strong intramolecular hydrogen bond (OH to N) but now the configuration at C-12 is changed to a trans ring system. Because of this change in configuration most of the nuclei are in new environments. Expected positions for the resonances are:



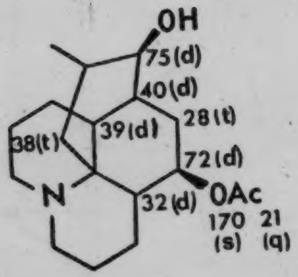
Positions 3, 5, 15 and 16 are expected to be the same as in lycopodine. C-1 is expected to be deshielded by about 3.6 p.p.m. on changing from a cis to a trans ring system, likewise C-9 no longer experiences the γ_{2HH} interaction and is also deshielded by about 3.0 p.p.m. Indeed C-1 and C-9 have almost identical environments except for the hydroxyl group δ to C-9. Because of this C-9 is shielded slightly and is expected 0.5 to 1 p.p.m. upfield from C-1. C-2 no longer has the γ_{HH} interaction and is expected to move about 5.5 p.p.m. downfield. C-4 no longer experiences a γ_{HH} and γ_{2HH} interaction but this steric interaction is replaced in part by that of a γ-hydroxyl group, hence it is expected about 3 p.p.m. downfield. C-6 should be almost unchanged, the loss of the γ_{HH} interaction being compensated for by the gain of a γ-hydroxyl interaction. C-7 is β to an axial hydroxyl group and is expected to move about 6 p.p.m. downfield. C-8 experiences a new γ_{HH} interaction shifting it upfield, while C-10 experiences the γ-hydroxyl interaction and is also shifted upfield by about 6 p.p.m. C-11 is β to the hydroxyl group and is expected to be deshielded by about 6 p.p.m. C-12 should be the same in "L-8" and "L-23" and is expected at about 70 p.p.m. C-13 should be deshielded by the adjacent hydroxyl group but this is counterbalanced by an increased steric crowding about this position, hence it is expected about 3 p.p.m. downfield. The most difficult position to estimate is that of C-14 since there are now two γ_{HH} and two γ_{2HH} interactions and good models are not available for this situation. The effect of two sets of interactions is usually greater than twice the value of a single interaction and C-14 is thus expected at around 30 p.p.m.

Using the calculated values the experimental results are assigned relatively easily. C-4 (d) which is assigned the 52.3 p.p.m. resonance is at considerably lower field than expected, inferring that the γ-hydroxyl has very little steric effect. C-14 is assigned the resonance at 25.6 p.p.m. which means the value of the two γ_{HH} + two γ_{2HH} interaction is about 24.5 p.p.m. This large value reflects the great steric crowding in the system which apparently cannot be relieved by slight deformation, as the C-15 and C-5 resonances also are upfield slightly from lycopodine.

The acidification experiment also produced anomalous results, C-14 shifting to lower field in this case, whereas in every other case it shifts to higher field. This is possibly a result of protonation of the nitrogen relieving the steric interaction about C-14 although this seems unlikely. Since the effect of the nitrogen lone pair is difficult to assess, perhaps it is unrealistic at this stage to attempt to rationalize this change.

A further point to emerge from the acidification experiment is that C-13 is still deshielded on protonation of the nitrogen. This deshielding cannot be due to interaction of nitrogen lone pair and carbonyl π orbitals since the configuration now precludes any σ coupled p interaction.

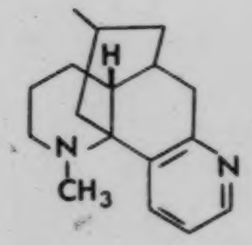
The final compound in the series is α-lofoline (31). The spectrum should be very similar to that of dihydrolycopodine, allowing for the axial hydroxyl group at C-8. Clavolonine also makes a useful model although the C-8 functionality is epimeric with that of α-lofoline. Resonances which differ from lycopodine are expected to be:



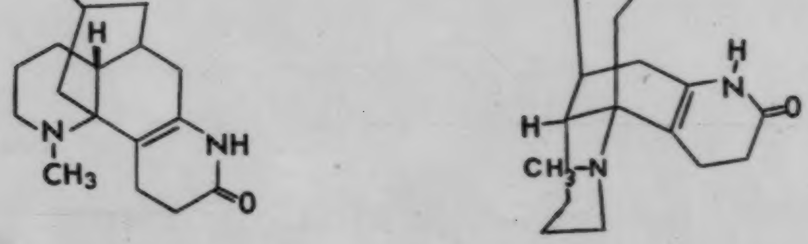
Using the methylcyclohexanols as models for comparing changes on going from an equatorial hydroxyl group to an axial hydroxyl group it is possible to calculate the resonances of carbons 7, 8, 12, 14 and 15. Clavolonine (28) was used as the standard model for this system. C-4 should be the same as in dihydrolycopodine, while C-5 should shift 3→4 p.p.m. downfield on acetylation. The equatorial proton at C-6 experiences a steric crowding from the acetoxyl group, comparable to a γ_a interaction and is expected about 6 p.p.m. upfield from its position in dihydrolycopodine. The resonance positions of acetoxyl group carbons are well known[48]. Hence the assignments can again be made as shown. As in earlier cases the C-5 and C-15 resonances appear upfield from the expected values reflecting the greater steric crowding caused by an acetoxyl group as compared to a hydroxyl group.

The preceding treatment has enabled the resonances of the carbon nuclei in an alkaloid of the lycopodine-type to be identified with a reasonably high degree of confidence. Should a new alkaloid of this type be isolated in sufficient quantity c.m.r. would now be a useful tool in its structure elucidation. Similarly it becomes a useful method for studying the stereochemistry of alkaloids of known constitution but undetermined stereochemistry. A problem of this type exists in the case of the alkaloid sauroxine. The structure and stereochemistry of α-obscurine (33) is known with certainty[49]. Sauroxine (34), which must be a stereoisomer of α-obscurine, has been assigned the 12-*epi* configuration, mainly on the basis of mass spectrometric and p.m.r. results (Chart 4)[50]. In order to confirm this assignment, we have studied their c.m.r. spectra, along with that of N-methyllycodine (32). These could then be compared to the spectrum of lycodine, the best available model for this system; the other model used was 2-piperidone[51] (Chart 3).

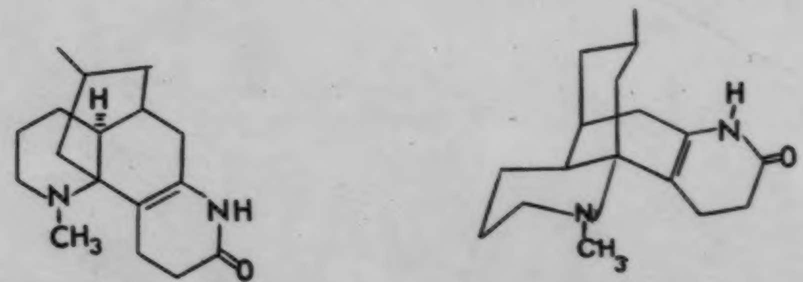
The results obtained for lycodine (3),



N-methyllycodine (32)



α-obscurine (33)

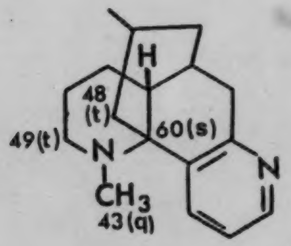


sauroxine (34)

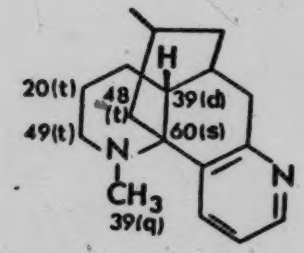
CHART 4. The configuration of N-methyllycodine, α-obscurine and sauroxine.

N-methyllycodine (32), α-obscurine (33), and sauroxine (34) are summarized in Table 3. The assignment of these shifts is discussed below. Acidification data is recorded in Table 4.

The spectrum of N-methyllycodine is expected to be very similar to that of lycodine, positions which would be changed by addition of the methyl group are:



A useful model for this system was N-methyl-2-methylpiperidine (Chart 3)[52]. These predictions assume that the N-methyl group is in the equatorial configuration and the results clearly show that this cannot be the case. However, assuming an axial configuration for the N-methyl group, the predicted shifts for N-methyllycodine are shown below:



This is in much better agreement with the experimental results and clearly indicates that the N-methyl group is predominantly axial. In particular the C-12 signal is an easily identified doublet which is shielded (relative to lycodine itself) by the methyl group by about 9 p.p.m. There is no other reasonable explanation for the origin of the high field signal at δ 19.8. The N-methyl signal is at unusually high field (36.4 p.p.m.).* These shifts show that the N-methyl group is interacting strongly with C-10 and C-12 and thus it must be axial. It is presumably the large C-17, C-3 interaction which forces the N-methyl group into the axial conformation. The slight downfield shift of C-3 in N-methyllycodine compared to lycodine itself shows the absence of a C-17, C-3 interaction in N-methyllycodine, which if present would have produced considerable shielding of the C-3 nucleus. In α-obscurine models show a reduction of this interaction as C-3 is now sp^3 hybridized. However it is not obvious from the models which conformation of C-17 will be preferred, axial or equatorial. The c.m.r. spectrum shows that again the axial conformation is preferred. The expected resonances for α-obscurine based on N-methyllycodine as model are:

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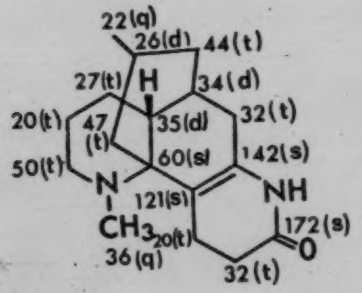
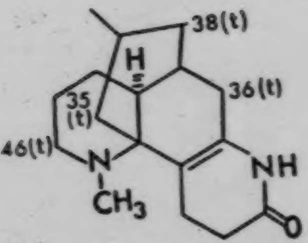
* Subsequent to this study Eliel and co-workers (J. Amer. Chem. Soc. 96 2257 (1974)) have provided an example of an axial N-methyl group which resonates at δ 33.27 p.p.m.



These are in good agreement with the observed values. As noted previously, change in configuration at C-12 (from the "normal" configuration ("L-8") to the 12-epi configuration ("L-23") causes a dramatic shift in the resonance position of C-14 (36.1 p.p.m.→ 25.6 p.p.m.). If sauroxine is indeed epimeric at C-12 with β-obscurine a similar shift should occur. The major changes expected in the spectrum of sauroxine from that of α-obscurine (assuming an axial N-methyl) are:



C-6 is deshielded, losing a γ_{HH} interaction as does C-9. C-14 experiences the greatest change, gaining both γ_{HH} and γ_{2HH} interactions. C-11 loses one interaction but gains another and so the shift should therefore remain constant. These predicted values are in good agreement with the measured values.

These c.m.r. results thus are in full agreement with the assignment of configuration at C-12 in the α-obscurine-sauroxine pair. In addition they provide the first evidence that in these alkaloids, as well as in N-methyllycodine, the preferred configuration at the nitrogen is that with the methyl group axial.

A further use for the results presented in this section might be in the study of the biosynthesis of these alkaloids, utilizing biosynthetic precursors enriched in ^{13}C.

TABLE 1

Carbon 13 Chemical Shifts[a] of Some *Lycopodium* Alkaloids

	1	25	26	27	28	29	30	31	3
C-1	47.2	47.1	47.2	46.2	47.2	46.2	48.7	46.9	147.1
C-2	19.3[b]	20.7	19.9	16.9	18.7[b]	17.8[b]	25.2[h]	18.1[b]	121.6
C-3	20.1[b]	23.5[f]	24.1[f]	25.8[f]	19.4[b]	19.8[b,g]	18.7[g]	20.3[b]	133.4
C-4	43.4	32.6	37.2	79.8	43.1	43.3	52.3	31.1	136.2
C-5	213.4	68.5	71.5	211.0	213.2	212.1	210.5	70.2	158.8
C-6	43.3	34.0	36.6[e]	39.6	36.5	44.4	44.0	29.3	35.5
C-7	37.3	35.6	36.3[e]	36.8	43.1	40.8	40.9	42.8	34.1
C-8	43.1	42.0	42.2	43.0	78.9	35.8	37.5	75.2	41.5[i]
C-9	47.8	47.4	47.2	49.4	47.2	46.2	48.0[d]	47.2	44.1[i]
C-10	26.4[c]	26.6[c,f]	26.4[c,f]	27.4[c,f]	26.2[c]	20.6[g]	21.0[g]	26.4[c]	26.4[c]
C-11	25.7[c]	25.0[c,f]	24.8[c,f]	25.8[c,f]	25.1[c]	30.3	30.3	24.2[c]	28.2[c]
C-12	45.5	45.8	45.5	46.2	43.9	69.5	70.1	37.4	44.9
C-13	59.9	55.2	56.8	59.0	59.7	61.6	62.5	54.6	56.2
C-14	43.7	43.2	43.4	44.8	42.0	36.1	25.6[h]	38.3	51.7
C-15	25.8	23.5	28.9	25.5	33.0	24.3	24.6	27.5	25.9
C-16	23.3	24.1	24.1	23.2	19.4	22.6	22.6	23.0	22.1
$\overset{O}{\overset{\|}{C}}-\overset{*}{C}H_3$								21.4	
$O{=}\underset{*}{C}{-}CH_3$								170.20	

a. Accurate to ±0.05 p.p.m. from internal TMS. Higher positive numbers indicates a decrease in shielding.

b. C-2 and C-3 may be reversed.

c. C-10 and C-11 may be reversed

d. C-1 and C-9 may be reversed.

e. C-6 and C-7 may be reversed.

f. C-3, C-10 and C-11 may be reversed.

g. C-3 and C-10 may be reversed.

h. C-2 and C-14 may be reversed.

i. C-8 and C-9 may be reversed.

TABLE 2

N-Protonation Shifts[a] ($\delta^{13}C_{ACID} - \delta^{13}C_{BASE}$) of Some *Lycopodium* Alkaloids

	1[b]	25[c]	26[c]	27[c]	28[d]	29[d]	30[d]	31[c]	3[c]
C-1	-0.8[e]	-0.9	-0.7	0.3	-0.6	-0.2	-0.5	-0.8	0.6
C-2	-0.8	-1.1	-0.9	-1.2	-0.7	-0.2	-2.8	-0.2	1.3
C-3	-1.9	-1.8	-2.2	-1.3	-1.6	-1.8	-1.1	-1.0	0.4
C-4	1.2	1.2	1.2	-2.1	1.1	2.4	-2.6	0.8	-3.8
C-5	-5.4	-2.8	-1.8	-3.1	-5.0	-5.0	-2.9	-1.0	-0.6
C-6	-0.5	-0.4	-0.8	-0.4	-0.6	-0.5	-0.4	-0.7	-0.8
C-7	-0.7	-0.3	-0.7	-0.4	-0.6	0.5	0.9	-0.3	-0.9
C-8	-1.6	-1.3	-1.4	-1.3	-2.2	-0.8	-0.4	-1.6	-1.1
C-9	-0.7	-1.1	-0.9	0.6	-0.7	-0.2	-0.1	-1.1	-2.0
C-10	-3.4	-3.2	-3.4	-2.3	-3.2	-2.7	-2.5	-2.5	-1.9
C-11	-2.1	-1.6	-2.1	-2.5	-1.8	-2.0	-1.5	-2.9	-2.5
C-12	-2.8	-2.4	-2.5	-1.9	-2.8	0.0	0.0	-2.2	-1.9
C-13	3.8	6.7	6.0	3.4	3.4	4.5	4.8	4.7	2.8
C-14	-4.5	-4.3	-4.6	-3.1	-4.4	-3.3	3.8	-3.7	-3.1
C-15	0.2	-0.2	-0.1	0.1	-0.4	0.3	-0.5	0.2	-0.8
C-16	-0.8	-0.7	-0.7	-0.4	-0.7	-0.8	-0.4	-0.2	-0.4
$\overset{O}{\overset{\|}{C}}\text{-}\overset{*}{C}H_3$								0.0	
$\overset{O}{\overset{\|}{^{*}C}}\text{-}CH_3$								-0.2	

a. Positive algebraic numbers indicate a decrease in shielding.

b. Carbon 13 titration curve determined experimentally (0.5 M).

c. Connectivity of carbon resonances of free base to the acidified solution done by analogy.

d. Carbon 13 titration curve determined experimentally (0.22 M).

e. See Table 1 for ambiguous assignments.

TABLE 3

Comparison of the Carbon 13 Chemical Shifts[a] of α-Obscurine and Sauroxine

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	3	32	33	34
C-1	147.1	146.7	171.8	171.6
C-2	121.6	121.6	30.4	31.1
C-3	133.4	134.4	19.5	18.6[f]
C-4	136.2	138.1	114.2	115.4
C-5	158.8	158.4	130.6	132.0
C-6	35.5	35.3[d]	31.4	35.2[g]
C-7	34.1	34.0	33.7	32.7[h]
C-8	41.5[c]	44.0	43.6[e]	36.3[g]
C-9	44.1[c]	50.5	51.4	47.7
C-10	26.4[b]	19.8	19.5	19.7[f]
C-11	28.2[b]	27.1	26.5	25.0
C-12	44.9	34.7[d]	36.0	33.7[h]
C-13	56.2	59.7	59.0	59.6
C-14	51.7	48.3	43.0[e]	35.2[g]
C-15	25.9	26.5	27.2	27.7
C-16	22.1	22.4	22.3	22.5
C-17		36.4	35.6	36.6

a. Chemical shifts accurate to ± 0.1 p.p.m. from internal TMS. Higher positive numbers indicate a decrease in shielding.

b. C-10 and C-11 may be reversed.

c. C-8 and C-9 may be reversed.

d. C-6 and C-12 may be reversed.

e. C-8 and C-14 may be reversed.

f. C-10 and C-3 may be reversed.

g. C-6, C-14 and C-8 may be reversed.

h. C-7 and C-12 may be reversed.

TABLE 4

N-Protonation Shifts[a] ($\delta^{13}C_{ACID} - \delta^{13}C_{BASE}$)

of α-Obscurine and Sauroxine

	3[c]	32[b]	33[c]	34[b]
C-1	0.6[d]	0.0	0.6	0.8
C-2	1.3	1.1	-0.5	-0.8
C-3	0.4	0.9	-0.4	-0.6
C-4	-3.8	-3.1	-5.4	-5.6
C-5	-0.6	-1.0	2.8	3.7
C-6	-0.8	-1.2	-0.9	-0.2
C-7	-0.9	-0.7	-0.9	2.4
C-8	-1.1	-0.7	-0.9	-0.9
C-9	-2.0	-0.3	-0.8	-0.3
C-10	-1.9	-0.8	-1.1	-0.1
C-11	-2.5	-1.4	-1.8	-1.8
C-12	-1.9	0.8	0.4	-0.8
C-13	2.8	3.7	5.7	6.8
C-14	-3.1	-2.1	-2.5	-2.7
C-15	-0.8	0.0	-0.1	0.0
C-16	-0.4	-0.4	-0.6	-0.7
C-17		-0.6	-0.4	-2.1

a. Positive algebraic numbers indicate a decrease in shielding.

b. Carbon 13 titration curve determined experimentally.

c. Carbon 13 titration curve determined by analogy.

d. See Table 3 for ambiguities.

EXPERIMENTAL

R_f value = distance moved by compound/distance moved by solvent.

Melting points were determined on a Fischer-Johns hot-stage melting point apparatus and are uncorrected.

Infrared spectra were recorded on a Perkin-Elmer Model 421 dual grating infrared spectrophotometer

Proton magnetic resonance spectra were measured using a Varian Associates Model HR-100 spectrometer with tetramethylsilane as standard.

C. m. r. spectra were determined at 22.63 MHz in the Fourier mode using a Bruker HFX-90 spectrometer in conjunction with a Nicolet-1085, 20 K mercury computer, or at 25.1 MHz on a Varian HA-100-15 system interfaced to a Digilab FTS/NMR 3 Data System and associated pulse and decoupling units.

Mass spectra were recorded on an A. E. I. Model MS-9 mass spectrometer or an A. E. I. Model MS-2 mass spectrometer.

O. r. d. and c. d. spectra were determined on a Jasco o. r. d./c. d., SS-20-2 (modified).

U. v. spectra were recorded on Cary Model 15 u. v. and visible spectrometer.

[A] The Structure of Lycoflexine

Isolation of bases of *Lycopodium alpinum*

L. alpinum, collected in the region of Jasper, Alberta, was air-dried and ground to give 4.5 kg. of fine powder. The powder was extracted in a Soxhlet apparatus, with commercial grade methanol, in 600 g. batches each for 72 hours. The methanolic solution was concentrated under reduced pressure to a thick sludge. The combined residues were stirred vigorously with 5% hydrochloric acid (3 l.) and the liquid decanted. The acidic solution was extracted with ether until no more material dissolved in the ether. The aqueous phase was then cooled to 0° C and sodium hydroxide solution (10 *M*) was added with rapid stirring until the solution was at pH 10-11. The aqueous phase was immediately extracted with chloroform until no more material dissolved in the chloroform. Concentration of the chloroform solution under reduced pressure yielded 8.5 g. of crude bases.

Isolation of bases of *Lycopodium clavatum* var. *inflexum*

The partially concentrated methanol extract of *L. clavatum* var. *inflexum* (from the Transvaal region of

South Africa) was obtained from Dr. B. Altenkirk of the South African Medical Research Council. The extract was concentrated to a thick sludge and the crude base mixture obtained in exactly the same way as from *L. alpinum*.

Isolation of Lycoflexine

The mixture of bases was dissolved in a minimum volume of benzene:chloroform (1:1) and chromatographed over alumina (grade II). The alkaloids were eluted with benzene, chloroform and methanol. The chloroform fraction was concentrated and rechromatographed over alumina again eluting with the same solvents. The chloroform fraction again was concentrated and chromatographed a third time over alumina, eluting with benzene, benzene/chloroform (1:1) and chloroform. Pure lycoflexine, m.p. 130 - 131°, R_f=0.37 (ethyl acetate/alumina t.l.c. plates) was crystallized from the concentrated benzene/chloroform fraction. The alkaloid mixture from *L. clavatum* var. *inflexum* contains approximately 3.5% lycoflexine whereas the *L. alpinum* extract contains only about 1.5% lycoflexine.

Treating lycoflexine with 70% perchloric acid in acetone (1:3) resulted in the precipitation of the perchlorate salt, m.p. 200 - 201°, which was recrystallized from acetone.

Treatment of lycoflexine with acetic anhydride and pyridine at room temperature for 24 hours yielded only starting material.

The i.r. spectrum ($CHCl_3$) of lycoflexine is shown in Fig. 2, the mass spectrum in Fig. 3. The u.v. spectrum shows absorption at λmax 230 nm (ε1380) and 298 nm (ε220). The p.m.r. spectrum (CD_3CO_2D) is shown in Fig. 5, the c.d. and o.r.d. spectra in Fig. 6 and Fig. 7.

Other alkaloids isolated from L. alpinum and identified by comparison with authentic samples include lycopodine, clavoline, lycodine and des-N-methyl-α-obscurine. Those isolated from L. clavatum var. inflexum include lycopodine and dihydrolycopodine.

Reduction of Lycoflexine

Lycoflexine (12 mg.) was treated with sodium borohydride (10 mg.) in methanol for 5 hours at room temperature. The solution was concentrated, diluted with hydrochloric acid to pH 3, then the solution was neutralized with 10% sodium bicarbonate solution. The aqueous solution was extracted with chloroform and concentrated to yield 9 mg. of dihydrolycoflexine, R_f=0.16 (ethyl acetate, alumina t.l.c. plates).

I.r.$_{(CHCl_3)}$: 3620, 1725, 1020 cm^{-1}.

C.d.: (c 0·0061 M, MeOH) 292 nm (Δε-2.24), 232 nm (Δε+3.10), (on acidification the band at 232 nm disappears and the band at 292 nm becomes positive (Δε+0.40).

Formation of Lycoflexine Methiodide

Lycoflexine (30 mg.) was dissolved in acetone (0.5 ml.) and three drops of methyl iodide added. The solution was left at room temperature and the methiodide salt precipitated quantitatively from solution. Recrystallization from chloroform (containing 0.75% ethanol) gave slightly yellowish crystals, m.p. 243 - 244° C. The i.r. spectrum ($CDCl_3$) shows carbonyl absorption bands at 1740 and 1700 cm^{-1}. The p.m.r. spectrum (D_2O) is shown in Fig. 8.

Hofmann Reaction of Lycoflexine Methiodide[53]

Lycoflexine methiodide (23 mg.), potassium t-butoxide in t-butyl alcohol (2 ml., 0.88 M), t-butyl alcohol (1 ml.) and benzene (2 drops) were heated under reflux for four hours. Water (5 ml.) was then added and the product extracted with chloroform. The reaction product was a mixture of four compounds, each different from the starting material, with R_f values of 0.88, 0.76, 0.65 and 0.10 (ethyl acetate, alumina t.l.c. plates). The compound with R_f=0.65 was the major product and was purified by chromatography over alumina (eluting with benzene/ether, 9:1) to yield 5 mg. of noncrystalline product. The mass spectrum is shown in Fig. 9.

Lycoflexine methiodide (50 mg.) and potassium t-butoxide (90 mg.) were dissolved in t-butyl alcohol (3 ml.) and benzene (5 drops) and heated under reflux for six hours then left at room temperature overnight. Water (5 ml.) was added and the solution extracted with chloroform. Concentration of the chloroform and recrystallization of the product from acetone yielded 15 mg. of product, m.p. 168° - 170°, R_f=0.23 (methanol/ethyl acetate 2:1 on alumina t.l.c. plates). This compound decomposed within three days at room temperature. The mass spectrum is shown in Fig. 10. The u.v. spectrum (methanol) showed an absorption at λmax 280 nm. (ε 5,600); in the presence of one drop of sodium hydroxide solution the absorption shifts to λmax 293 nm (ε 8,900).

I.r.$_{(CHCl_3)}$: 3500, 2800, 1715, 1675 cm^{-1} (Fig. 11).

Oxidation of Lycoflexine

Lycoflexine (11.5 mg.) and potassium t-butoxide (15 mg.) were dissolved in t-butyl alcohol (5 ml.) and oxygen was bubbled through the solution for 2.5 hours at room temperature. Water (10 ml.) was added and the solution extracted with chloroform. The material obtained by concentration of the chloroform solution was chromatographed over alumina, the reaction product eluting with 1-propanol. Four mg. of product were obtained R_f=0.12 (ethyl acetate, alumina t.l.c. plates). The mass spectrum is shown in Fig. 13. I.r.$_{(CHCl_3)}$: 3500, 1720, 1680 cm^{-1}. U.v. (methanol) λmax 283 nm (ε 5,900)

Deuteration of Lycoflexine

Lycoflexine (25 mg.) and phosphorus pentachloride (50 mg.) were added to deuterium oxide (0.5 ml.) and the solution was stirred for five days at room temperature. Excess anhydrous sodium carbonate was added and the solution extracted with chloroform, concentration of the chloroform extract yielded deuterated lycoflexine (22 mg.). The mass spectrum showed peaks at m/e 280; 279; 278; 277; 276; 275 in the ratio 1:3:8:9:4.5:1. Exact mass of the ion with m/e 280 corresponds to that of $C_{17}H_{20}NO_2D_5$. (Calculated 280.2277, measured, 280.2270). The n.m.r.

spectrum (CD_3CO_2D) is shown in Fig. 14.

Synthesis of Lycoflexine from Fawcettimine (experiment performed by Dr. Y. Fukazawa.)

Fawcettimine was treated with two equivalents of formaldehyde in methanol in the presence of a trace of hydrogen bromide, at 65° C for 24 hours. The solution was concentrated to give a mixture of four components as shown by t.l.c. The major component, comprising about 50% of the mixture, had an R_f value identical with that of lycoflexine. Preparative t.l.c. on alumina plates yielded the major compound identical in infrared spectrum, mass spectrum and t.l.c. behaviour with lycoflexine.

[B] Hydroxylycopodine lactam

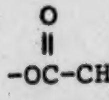
Isolation of 6-Hydroxylycopodine lactam

The alkaloid mixture from *L. clavatum* var. *inflexum* (supplied by Dr. B. Altenkirk, South African Medical Research Council) was chromatographed over alumina (grade II). Hydroxylycopodine lactam begins to elute with chloroform-methanol (9:1) and the bulk is then eluted with methanol. This yields a brown solid from which hydroxylycopodine lactam is obtained as white crystals by recrystallization from chloroform. Final purification is effected by sublimation at 150° (0.05 mm.) which yields a fine white crystalline powder, m.p. 200 - 202.5° C. This compound shows R_f=0.46 (alumina t.l.c. plates, acetone-methanol 9:1); mass spectrum m/e 277 ($C_{16}H_{23}NO_3$, 20%); 220 ($C_{12}H_{14}NO_3$, 100%); 192 ($C_{11}H_{14}NO_2$, 10%); 85 (50%); 83(60%) as in Fig. 18.

I.r.$_{(CHCl_3)}$: 3400, 2900, 1700, 1630, 1460 cm^{-1}; as in Fig. 19.

P.m.r. ($CDCl_3$): δ 0.78 (d, 3, J=6c.p.s. $C\underline{H}_3$-CH) and δ 4.22 (m, 1, C$\underline{H}$-OH) Fig. 20.

Acetylation of Hydroxylycopodine lactam

Hydroxylycopodine lactam (20 mg.) dissolved in acetic anhydride (1 ml.) containing pyridine (1 drop) were stirred overnight at 0° C. The solution was concentrated under high vacuum to leave a solid residue which after recrystallization from acetone yielded a white crystalline solid m.p. 190 - 192°. R_f=0.51 (alumina t.l.c. plates, ethyl acetate); mass spectrum m/e 319 ($C_{18}H_{25}NO_4$, 15%); 262 ($C_{14}H_{16}NO_4$, 50%); 259 ($C_{16}H_{21}NO_2$, 45%); 220 (24%), 202 ($C_{12}H_{12}NO_2$, 100%). I.r.$_{(CHCl_3)}$: 1730, 1695, 1630 cm^{-1} (Fig. 21). P.m.r. ($CDCl_3$): δ 0.88 (d, 3, J=6c.p.s. C$\underline{H}_3$-CH); δ 2.08 (s, 3,  $-O\overset{\overset{O}{\|}}{C}-CH_3$); δ 2.94 (t(d), 1, J=14c.p.s. and 4c.p.s., N-C$\underline{H}$); δ 4.50 (d(d), 1, J=14c.p.s. and 4c.p.s., N-C$\underline{H}$); δ 5.40 (m, 1, C$\underline{H}$(OAc)), Fig. 22.

Diacetylation of Hydroxylycopodine lactam

The monoacetate (5 mg.) was refluxed with acetic anhydride (0.5 ml.) and pyridine (0.1 ml.) for four hours. The solution was concentrated under high vacuum to yield an oil R_f=0.68 (alumina t.l.c. plates, ethyl acetate); mass spectrum m/e 361 ($C_{20}H_{27}NO_5$, 8%); 319 ($C_{18}H_{25}NO_4$, 12%);

304 ($C_{16}H_{18}NO_5$, 6%); 301 ($C_{18}H_{23}NO_3$, 6%); 262 ($C_{14}H_{16}NO_4$, 48%), 259 ($C_{16}H_{21}NO_2$, 62%); 220 (29%), 202 ($C_{12}H_{12}NO_2$, 100%).

Chlorination of Hydroxylycopodine lactam

Hydroxylycopodine lactam (20 mg.), thionyl chloride (1.5 ml.) and dichloromethane (8 ml.) were stirred at room temperature for 40 hours. The solution was concentrated to dryness and the product recrystallized from acetone to yield 17 mg. of white crystalline product, m.p. 102 - 105°. R_f=0.74 (alumina t.l.c. plates, ethyl acetate); mass spectrum m/e 295 ($C_{16}H_{22}NO_2{}^{35}Cl$, 2%); 260 (2%); 238 ($C_{12}H_{13}NO_2{}^{35}Cl$, 12%); 210 (2%), 96 (35%); 81 (15%); 79 (100%).

I.r.$_{(CHCl_3)}$: 1700, 1635 cm^{-1} as in Fig. 23.

P.m.r. ($CDCl_3$) as for hydroxylycopodine lactam except there is no signal at δ 4.22, (Fig. 28).

Attempted Mesylation of Hydroxylycopodine lactam

Hydroxylycopodine lactam (22 mg.) and methanesulphonyl chloride (0.25 ml.) in pyridine (3 ml.) were allowed to react for 2½ hours at 0° C, after which time considerable darkening of the reaction mixture had occurred. Evaporation of the solvent and recrystallization

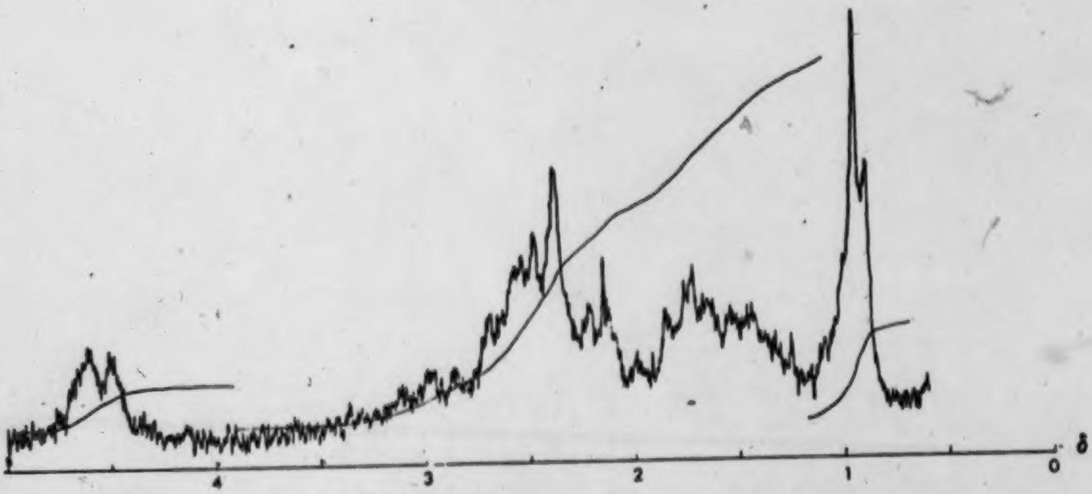


Fig. 28 The p. m. r. spectrum of chlorolycopodine lactam.

of the product from acetone yielded 15 mg. of the chlorolycopodine lactam obtained previously.

Attempted Dechlorination

Chlorolycopodine lactam (5 mg.) was stirred with pyridine (1 ml.) at room temperature for two hours without change. Heating under reflux for six hours produced a black tar. After column chromatography over alumina (grade III) the only product which was obtained was unchanged starting material.

Chlorolycopodine lactam (5 mg.), sodium methoxide (30 mg.) and methanol (5 ml.) were stirred for 22 hours. Water (10 ml.) was added and the reaction mixture extracted with chloroform. Evaporation of the chloroform yielded only unchanged starting material.

Dechlorination with zinc

Chlorolycopodine lactam (20 mg.), glacial acetic acid (1.5 ml.) and zinc dust (50 mg.) were heated under reflux for 22 hours. The zinc was filtered and the filtrate diluted with water (20 ml.) and extracted with chloroform. Concentration of the chloroform yielded a solid with R_f=0.45 (alumina t.l.c. plates, ethyl acetate)

identical with that of lycopodine lactam. Mass spectrum m/e 261 (20%), 204 (100%), 176 (25%), 83 (100%).
I.r.$_{(CHCl_3)}$: 1700, 1620 cm^{-1}.

Dechlorination with Boron trifluoride etherate

Chlorolycopodine lactam (10 mg.) and boron trifluoride etherate (0.1 ml) in ether (0.3 ml.) were combined with a solution of lithium iodide (0.05 g.) in ether (1 ml.), under nitrogen at room temperature. Two drops of chloroform were added to the mixture to increase the solubility of chlorolycopodine lactam. After stirring for 44 hours, sodium sulphite (10% solution) was added to destroy the iodine formed and the mixture was extracted with chloroform. The mixture obtained after concentration of the chloroform was purified by chromatography over alumina (grade I). Lycopodine lactam (5 mg.) was obtained by elution with chloroform. R_f=0.45. I.r. and mass spectra were identical with those of lycopodine lactam and those of the compound obtained by zinc/acetic acid dechlorination.

[C] Carbon 13 n.m.r. of some Lycopodium Alkaloids

All the undeuterated alkaloids are naturally occurring and were available from previous work in these laboratories. The deuterated compounds were prepared in cooperation with Dr. L. M. Browne as described below.

Lycopodine lactam (prepared by the method of Law[54])

Lycopodine (1.0 g.) was dissolved in acetone (50 ml.) and potassium permanganate (0.6 g.) added in portions over three hours. The solution was filtered and concentrated. The residue was redissolved in acetone and potassium permanganate (0.6 g.) added over a two hour period. The solution was filtered and concentrated. The residue was dissolved in buffer of pH 4 and extracted with ether. Concentration of the ether extract gave a mixture of lycopodine and lycopodine lactam which was separated by chromatography over alumina. Recrystallization of the lactam yielded 0.18 g. of product with similar i.r. spectra and m. p., 163° C, to that previously reported.

Lycopodine-9,9-$\underline{d}_2$

Lycopodine lactam (0.18 g.) in ether (5 ml.) was added dropwise to a stirred, cooled solution of lithium aluminium deuteride (0.06 g.) in ether-dimethoxyethane (25:1). The reaction mixture was heated under reflux for four hours, then cooled. Excess lithium aluminium deuteride was destroyed by the addition of water (2 drops), 5% sodium hydroxide solution (6 drops) and water (2 drops). The precipitate was filtered and washed with methylene chloride. The combined organic fraction was dried (anhydrous magnesium sulphate) and concentrated, yielding 0.13 g. dihydrolycopodine-5,9,9-$\underline{d}_3$ (as shown by low resolution mass spectroscopy). Dihydrolycopodine-5,9,9-$\underline{d}_3$ (0.13 g.) was dissolved in acetone (5 ml.) and cooled to 0° C. Jones reagent (8$\underline{N}$, 10 drops) was added. The organic solution was decanted, concentrated and diluted with 5% sodium hydroxide solution. This was extracted with methylene chloride. Concentration yielded 0.12 g. lycopodine-9,9-$\underline{d}_2$ (as shown by low resolution mass spectroscopy).

Lycopodine-4,6,6-$\underline{d}_2$,$\underline{d}_3$

Lycopodine (0.16 g.) was dissolved in deuterium oxide (1 ml.) and phosphorus pentachloride (1 g.) was added cautiously. The stoppered reaction mixture was allowed to stand at room temperature for five days then basified by careful addition of anhydrous sodium carbonate. The mixture was diluted with deuterium oxide, extracted with ether, the ether extract dried (anhydrous magnesium sulphate) and concentrated to give 0.135 g. of a mixture lycopodine-4,6-$\underline{d}_2$ and lycopodine-4,6,6-$\underline{d}_3$ (1:1) as shown by low resolution mass spectrometry.

Dihydrolycopodine-4,6,6-$\underline{d}_2$,$\underline{d}_3$

Lycopodine-$\underline{d}_2$-lycopodine-$\underline{d}_3$ (0.13 g.), anhydrous sodium carbonate (trace) and sodium borohydride (0.1 g.) were dissolved in deuteromethanol. The reaction mixture was allowed to stand at room temperature overnight, then diluted with aqueous hydrochloric acid. The aqueous extract was washed with ether, basified with 10% sodium hydroxide solution, then extracted with methylene chloride. The methylene chloride extract was dried (anhydrous magnesium sulphate) and concentrated to give 0.125 g. dihydrolycopodine-4,6,6-$\underline{d}_2$,$\underline{d}_3$ (1:1) as shown by low resolution mass spectrometry.

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II : AN INVESTIGATION
OF THE SWIMMING RESPONSE
OF THE SEA ANEMONE
STOMPHIA COCCINEA
TO CERTAIN STARFISH

INTRODUCTION

In 1955 an unusual behavior pattern, a so-called "swimming response" was described in the sea anemone Stomphia coccinea[1]. Normally sea anemones remain fastened to their substratum; when disturbed they respond by withdrawing the tentacles quickly and closing up the oral region, reopening slowly when the stimulus is withdrawn.

S. coccinea behaves normally to simple tactile stimulation; its swimming response occurs when it is brought into contact with certain species of asteroids. The basal disc shrinks while the body extends, it then detaches and propels itself through the water with a series of whip-like motions[2,3,4]. The anemone typically takes eight to ten seconds to detach. The swimming motion lasts for a few minutes, after a quiescent period lasting from five to fifteen minutes it reattaches. During this swimming period the anemone is insensitive to all except very gross tactile stimulation, but when ready to reattach it is sensitive again.

This behavior was subsequently further examined to see if it was in any way general, however it proved to be quite specific. The anemone S. coccinea was found to swim in response to the starfish *Dermasterias imbricata*, *Hippasterias spinosa* and *Hippasterias phrygiana*[5]. Of about 15 other species tested none have a significant effect. *Stomphia* was also found to swim when contact is made by the nudibrank *Aeolidia papillosa*[6]. Electrical stimuli also elicit the swimming response at certain intensities and frequencies of stimulation[7].

Touching a single tentacle of the anemone momentarily with the asteroid *Dermasterias* will produce a swimming response. The column is less sensitive, if a *Dermasterias* is placed over a closed *Stomphia* the anemone will usually detach but only after three or four minutes.

Until 1967 all the anemones reacting in this way were assigned to the single species, *Stomphia coccinea*. It had been noted that amongst these anemones two types existed: a so-called "small *Stomphia*" which corresponded with descriptions of *S. coccinea* from Europe [8], and a so-called "large *Stomphia*" differing in colour and markings as well as size which was also a much more active swimmer. In 1967 it was shown that "large *Stomphia*" swam when contacted by the "small *Stomphia*" and that the reciprocal test did not cause "small *Stomphia*" to swim[9].. The results suggested these were two different species.

The first opinion placed "large *Stomphia*" in the genus *Actinostola* but more recently it has been regarded as a new species of *Stomphia*, *S. didemon*[8], and this name will be used in this work. Like *S. coccinea*, *S. didemon* swims in response to the asteroids, the nudibranch and to electrical stimulation. Its swimming motion is much more vigorous than that of *S. coccinea* but otherwise very similar. *S. coccinea* will not cause *S. coccinea* to swim[9].

The reason for this response is not clear. *D. imbricata* is quite a fairly common starfish and occurs subtidally or in slightly deeper water off the west coasts of North America. *H. spinosa* occurs similarly but is much less common. *H. phrygiana* is a North Sea species. *S. coccinea* occurs typically at depths of greater than ninety feet, usually it is found on mussel shells and although it co-occurs with *S. didemon*, the latter is much less common. The anemones are obtained by dredging and only one *S. didemon* is obtained for every fifty to one hundred *S. coccinea*.

The distribution of these animals makes the "escape reaction" very puzzling. There seems to be no reason for *S. didemon* to escape from *S. coccinea*, indeed normally they would never come in contact with one another. Moreover *Stomphia* and *Dermasterias* should come into contact only very rarely and even then anemones do not form a normal part of the diet of asteroids. (However a *Dermasterias* has been observed "eating" a *Stomphia* in the laboratory.) The nudibranch *Aeolidia* has been observed to eat *Stomphia* but again this is in a laboratory aquarium and it is unknown if this behavior takes place in its natural environment[10]. Possibly there is a genuine escape response to the nudibranch and the stimulus that triggers this response is accidentally reproduced by the asteroid and, with respect to *S. didemon*, by *S. coccinea*.

When a cotton swab is rubbed on the surface of *Dermasterias* and the anemone's tentacles are touched with the swab the anemone will undergo a typical swimming reaction. Similar behavior is reported for *Aeolidia* rubbings, indicating that a definite compound or group of compounds is responsible for this behavior. Extracts of *Dermasterias* and *Aeolidia* will also elicit the swimming response. However rubbings or extracts of *S. coccinea* will not produce a swimming response from *S. didemon*, although this might be a concentration effect, *i.e.* extremely small amounts of material are concentrated in one part of the animal, or possibly the substance can not be extracted in the same way. Since a S. coccinea will cause S. didemon to swim but will not cause a second S. coccinea to swim the results indicate that there is more than one active substance; that contained in Dermasterias to which both anemones are sensitive and that contained in S. coccinea to which only S. didemon is sensitive.

The neuromuscular response of the anemones elicited in this way involves a complex series of movements and it is one of the fastest activities that anemones undergo. It would be strange if the anemones possessed the means for such a complex reaction, purely as an accidental feature, so one is inclined to assume that this behavior pattern is in some way important in the animal's life. The nature of the compound or compounds producing this reaction would help in the understanding of this response and the following discussion describes work directed towards isolating and identifying the active principle.

DISCUSSION AND RESULTS

Escape responses are well known in marine animals and escape responses to asteroids are common[11]. In particular, some clams and other bivalves frequently have a dramatic escape response: clapping the shells together to propel themselves backwards away from the starfish. The compounds that cause this response have been shown to be steroid glycosides[12] although a similar response can be produced by some other surface active materials such as detergents[13]. Stomphia coccinea has been shown to be insensitive to surfactants such as detergents[14]. Previous work has suggested that the active component of D. imbricata was not steroidal[15], so it seemed possible that this substance might be new to the field of marine escape responses.

The asteroids and actinaria were all obtained off the coast of the San Juan Islands, U.S.A. The asteroids were obtained by divers and the actinaria by dredging. This study was confined mainly to D. imbricata and S. coccinea, on the basis of availability. This was also fortuitous as D. imbricata appears to contain far more active material than H. spinosa.

Initially extracts of the active material were prepared by trisection of the starfish into the oral surface, the aboral surface and the gut and surrounding tissue. The coelomic fluid, itself active, was kept separate. The three sections were each homogenised with water and filtered through celite to give a cloudy solution. Repeated extraction of this solution with chloroform and further filtration through celite yielded a black, homogeneous aqueous solution.

The solution obtained from all three sections was found to be active. (The experimental section contains the detailed testing procedure and describes when the response to a solution is considered positive and hence the solution active.) This contradicts earlier work which stated that only the oral surface was active[15]. In our work, S. coccinea was found to swim to contact with either surface of the starfish and thus extracts from both surfaces are expected to be active.

As the active constituent seemed to be generally distributed in the starfish trisection was not used in the latter work.

Further experiments showed that even homogenisation of the tissue was unnecessary. The starfish was simply cut into approximately quarter inch slices and shaken for an hour with water. The water was decanted, the starfish pieces washed with acetone and then extracted by shaking with chloroform. The procedure was repeated a second, and if necessary, a third time. This method yields a black aqueous solution which is then filtered and freeze dried. The red chloroform solution is kept separate.

Yields seem to vary with the size of the starfish but typically a large D. imbricata which weighed 875 g. when wet contained 110 g. of coelomic fluid, which was added to the aqueous extracts. After freeze drying the total aqueous fraction there was 16.8 g. of water-soluble material (15% of total solids). The chloroform solution yielded 1.5 g. of material (1.6% of total solids). There remained 87 g. of dried insoluble material.

The red chloroform-soluble material was examined for steroids*. From the oily mixture, lathosterol (1)[16] was isolated fairly readily by column chromatography. Cholesterol (2)[17] was also isolated but in very small amounts.

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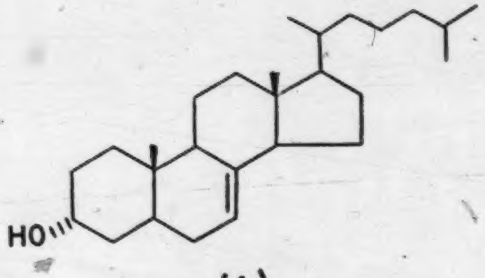
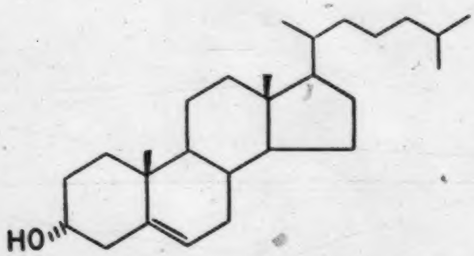
* Work done in conjunction with Dr. David King.



(1)



(2)

These steroids, identified by comparison with authentic samples, were the only steroids found in the mixture and the only compounds positively identified. The remainder of the chloroform-soluble material was a complex mixture containing long chain fatty acids, colouring material and other unidentified components. The chloroform-soluble material elicited no swimming response from S. coccinea.

The black, water-soluble powder from D. imbricata was active, eliciting swimming responses from S. coccinea when diluted to 0.5 to 0.1 mg/ml. At greater dilution there was very little swimming activity. During the course of the work a specimen of H. spinosa became available. H. spinosa was treated in the same manner as described for D. imbricata and tested in the same way. The compound producing the swimming response was either much less active or present in much lower quantities in *H. spinosa*, since the maximum dilution at which swimming activity was still produced was found to be around 50 mg/ml. Hence the work was restricted to *D. imbricata*. No further investigation was carried out on *H. spinosa* or any other starfish.

A few preliminary tests were carried out on the total crude aqueous mixture. The active substance was stable to boiling in water; after 30 minutes there was no apparent loss of activity. The freeze-dried powder was extracted with various solvents; the active substance is completely insoluble in chloroform, acetone and dry butanol. It is slightly soluble in methanol and moderately soluble in butanol saturated with water. An attempt was made to partition the crude, aqueous mixture using the water-butanol system, however the active substance was distributed between both phases and such techniques as counter-current distribution have not yet been pursued.

The crude aqueous solution gradually forms a precipitate during the first few weeks. This is a fine, black precipitate which was removed by centrifugation. This material is inactive and so was not investigated, presumably it is of proteinoid nature. The solution is a very good medium for fungus growth, when left at room temperature a noticeable growth of fungus usually formed on the surface within twenty-four hours. Both the fungal growth and precipitation problems can be prevented by storage in a deep freeze. Freezing has no noticeable deleterious effect on the activity.

Because of the dark colour of the crude aqueous mixture, colour tests were difficult to interpret. Dilution helps in this sense but considerably reduces the sensitivity.

Tests with ferric chloride were negative producing only a white precipitate. Tests for steroids[18] were inconclusive. Tests with ninhydrin[19] were also negative.

Many methods were investigated in an attempt to isolate the active substance from the total crude. It is completely involatile and much too polar to be eluted from silica gel, florisil, or alumina columns. The active material can be dialysed through a cellulose membrane. However virtually all of the total crude passes through the membrane and thus very little purification is effected in this way. Since the active substance is slightly soluble in methanol, attempts were made to fractionate the material by preferential dissolution in methanol. However, very little purification could be effected in this way. The use of Avicel (micro-crystalline cellulose) does produce some separation. It was used in column chromatography, preparative thin layer chromatography (p.t.l.c.) and high pressure liquid chromatography (h.p.l.c.). The active substance can be eluted from Avicel but the activity is greatly reduced and since a better procedure became available this method of separation has not been pursued. The most useful method of separation involves the use of Sephadex columns, either G10, G15 or G25 (cross-linked dextrans, exclusion sizes approximately 700, 1500 and 5000 respectively)[20].

The crude active solution is first centrifuged and then applied to the column, usually G15, and eluted with distilled water. The first fractions eluted are black, and foam readily. They contain higher molecular weight material, presumably some surface active material and possibly steroid glycosides. The following fractions are faintly yellow, support the growth of fungus and contain sugars and other compounds. Following these, there is a single fraction containing a golden-yellow compound. The column is then washed with several column volumes of distilled water. Testing each solution on S. coccinea showed that the activity was contained in the washings, i.e., the very last material to be eluted from the Sephadex column. Thus the substance eliciting the swimming response elutes after about three and a half column volumes whereas glucose, for example, elutes after two column volumes. The substance must be chemisorbed on the Sephadex suggesting that the substance is partially aromatic[21]. The active substance is chemisorbed to the same extent on G10, G15 and G25 (which were the three grades used) and for the same size column is eluted after the same volume in each case.

The active material collected in this way is brown-black in appearance and constitutes about 5% of the total weight of the water-soluble material. H.p.l.c. (methanol/cellulose) soon showed that this material is a mixture of at least six compounds. Unfortunately h.p.l.c. could not be used to fractionate the material as the active compound was not eluted from this system. Evidently more than the active substance is chemisorbed by Sephadex, suggesting the starfish produces several compounds of an aromatic nature. The active mixture eluted from Sephadex had activity at a dilution of 0.04 mg/ml indicating less than microgram amounts of the active compound will produce a swimming response.

These results show that a steroid glycoside is not responsible for the swimming activity since this type of compound would elute from Sephadex in a much smaller volume than found for the active material. Further, steroid glycosides from other starfish* produced no swimming responses from *S. coccinea*.

This active mixture, eluted from Sephadex, and henceforth called the crude concentrate was rather easier to work with than the total concentrate. In moderate concentration it forms a golden-yellow solution, foams much less readily, and no longer supports fungal growth. Testing this solution with ninhydrin produced no purple colouration, indicating the absence of free amino acids.

The ferric chloride test for phenolic compounds produced a brown precipitate. The solution remaining after the precipitate was removed did not elicit a swimming response from *S. coccinea*.

Treating the crude concentrate with charcoal decolourised the solution. The solution remaining was devoid of activity. Attempts to recover the active material from the charcoal were unsuccessful. This also suggests that the active constituent has a partial aromatic structure or one with extended conjugation[22].

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* Provided by Prof. J. ApSimon.

It was found that the crude concentrate could be fractionated by precipitation from methanol. The dried powder was dissolved in a large volume of dry methanol (e.g. one litre methanol for one hundred mg. of crude concentrate) and the insoluble material removed by filtration. As the solution was concentrated, a solid precipitated. The first fraction to precipitate was the most active, producing swimming responses at a dilution of 0.02 - 0.01 mg/ml.

However this procedure also did not give a clear cut separation. The activity of material dissolved in methanol was found to decrease with time and after a period of several weeks the activity was completely destroyed. Hence this method was not used further for fractionation.

Silylation[23] of the crude concentrate did not produce material soluble in acetone or chloroform. Similarly attempted acetylation with acetic anhydride and pyridine catalyst did not produce any chloroform soluble material. It did however deactivate the mixture. Treatment of the

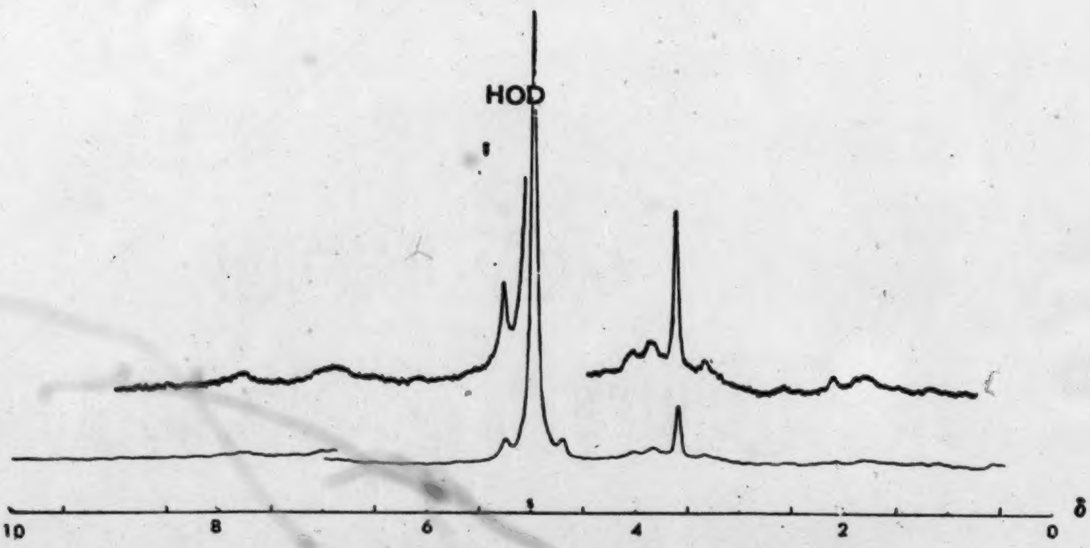


Fig. 1. The p. m. r. spectrum of the crude concentrate.

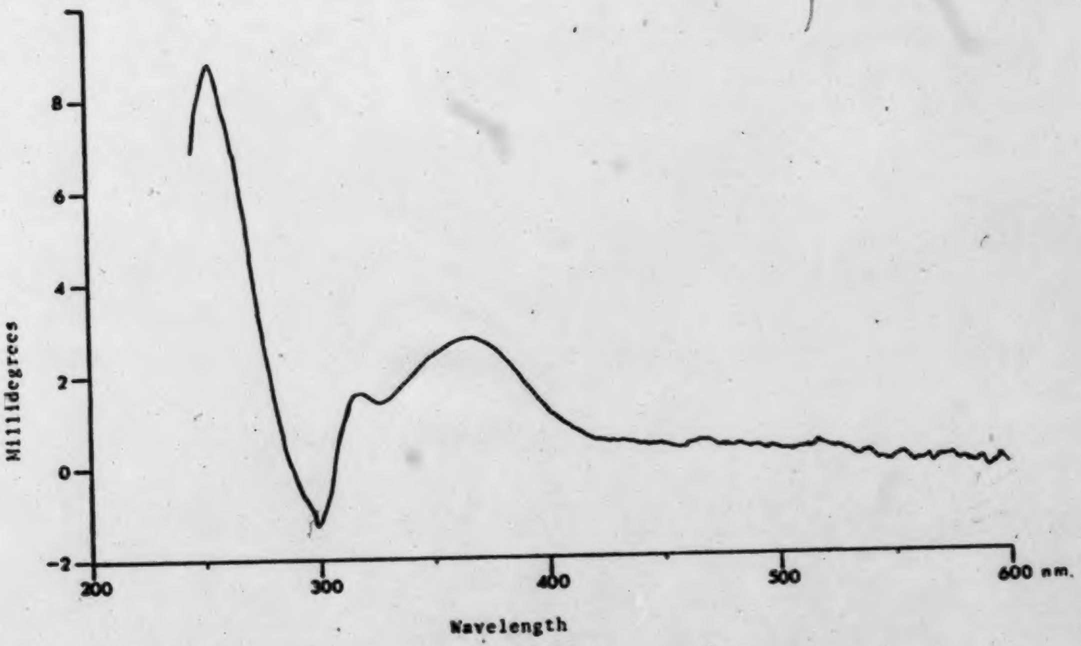


Fig. 2. The c. d. spectrum of the crude concentrate.

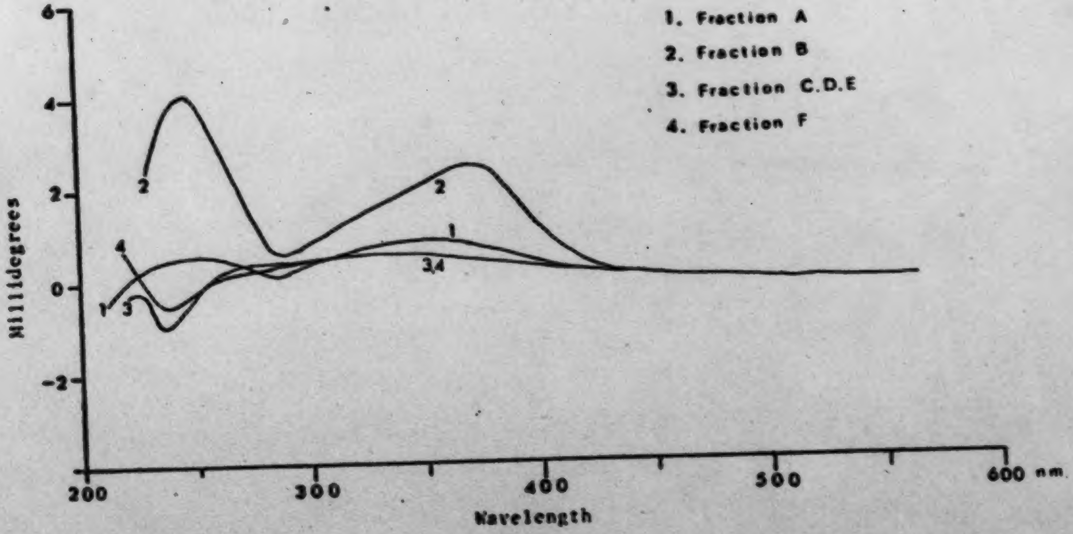


Fig. 3. The c. d. spectra of fractions obtained by methanol fractionation of the crude concentrate.

crude concentrate with dilute acid or base also quickly deactivates it. Attempted separation using ion-exchange resins was also unsuccessful. Mixed bed (*e.g.* Amberlite MB3), acid (*e.g.* Amberlyst A15) and base (*e.g.* Amberlyst A27) resins remove the active substance, either by absorbing it or decomposing it.

The non-ionic (polystyrene) resins XAD2 and XAD4[24] were investigated. Active material could be eluted from these with water-methanol mixtures. However there was a considerable loss of material with these resins and so they were not used for further separation.

It was not possible to obtain a mass spectrum from the crude concentrate, even at 300° C there were no volatile species detected. The i.r. spectrum was also not helpful. It indicated O-H and C-O stretching frequencies and little else. The p.m.r. spectrum (Fig. 1) was unusual. It showed a single strong sharp peak at δ 3.60 and practically no other resonances. This spectrum eliminated the possibility that steroids were present and was suggestive of the $-\overset{\oplus}{N}Me_3$ group[25]. Mayers test[26] for tetrasubstituted amines produced a brown precipitate (similar to that from ferric chloride) which was insoluble in acetone and methanol. Dragendorfs test[27] for basic nitrogen was negative. Both results are inconsistent with the presence of $-\overset{\oplus}{N}Me_3$. It was shown that the tetramethylammonium ion, present in some marine organisms[28] was not present, since it elutes much more quickly from Sephadex G15 than the crude concentrate.

The anemones were tested towards $R\overset{\oplus}{N}Me_3$ compounds. Tetramethylammonium chloride was found to be completely inactive. Choline and acetylcholine produced, in a few cases, a partial swimming response. These results might not be significant since massive concentrations (around 10 mg/ml) were necessary to produce a reaction.

The p.m.r. spectrum of material obtained after further purification of the crude concentrate no longer shows a signal at δ 3.60. Possibly the compound producing the peak is an inactive contaminant or the active material is partially decomposed on further purification.

The c.d. spectrum of the crude concentrate is shown in Fig. 2. Addition of acid or base to the crude concentrate reduces the c.d. spectrum to a straight line. The c.d. spectrum of each fraction obtained by methanol fractionation is shown in Fig. 3. Hence the active fraction seems to be associated with the chromophores at 255 nm and 360 nm. In the deactivated materials these absorptions are no longer present.

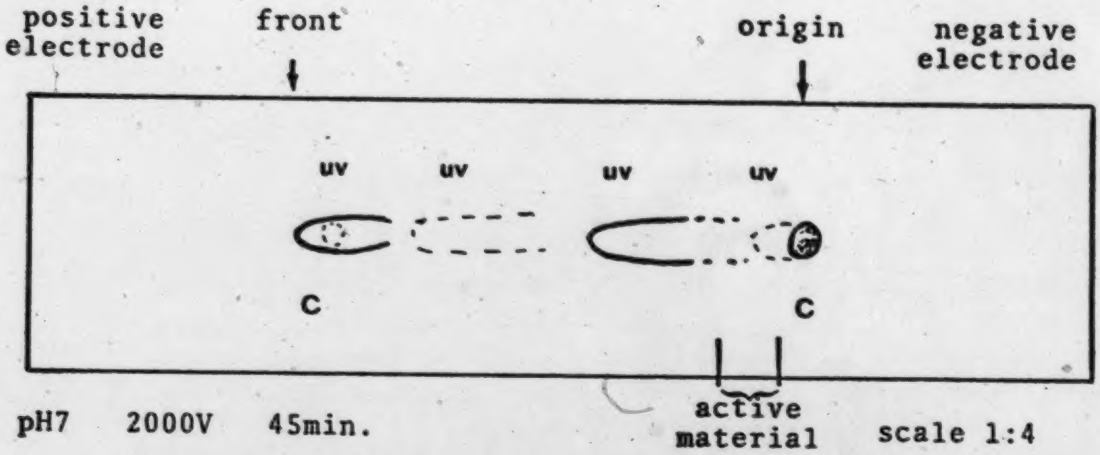
Analysis of the crude concentrate showed 30% C, 4% H and 8% N. No chlorine, iodine or sulphur was present. No significant amount of fluorine was present (by ^{19}F n.m.r.). Using X-ray fluorescence spectroscopy[29] 0.05% Br was detected in this mixture. However, further purification (see experimental) removed all the bromine; none occurs in the active fraction. E.s.r. spectroscopy[30] showed that the active fraction does not contain a transition metal.

An amino acid analysis[31] showed the crude concentrate was about 5% protein containing at least seventeen known amino acids, the major ones being iso-leucine, alanine, aspartic and glutamic acids and a large amount of tyrosine. The tyrosine probably accounts for the high molecular weight protein chemisorbing to the Sephadex. Earlier publications however suggest that the active component is not a protein[15].

Since the active substance was apparently ionic, electrophoresis[32] was next used to separate the constituents of the crude concentrate. Employing a phosphate buffer at pH7 a good separation was obtained as shown in Fig. 4. Surprisingly nearly all the components were negatively charged or neutral. On a preparative scale no positively charged species could be detected. By cutting the paper into strips and extracting the strips with water or methanol and testing the material thus obtained, the active substance was found in the range shown in Fig. 4, i.e., it is negatively charged and of low mobility. This was the only part of the chromatogram that contained activity, but the activity found was less than that found in the crude concentrate. This is possibly due to decomposition of the active substance under the conditions of the electrophoresis or because the filter paper adsorbs or modifies the compound irreversibly (as in the case of microcrystalline cellulose).

The active fraction obtained by this procedure again shows bands in the c.d. spectrum at 255 nm and 360 nm.

The electrophoresis separation procedure was repeated to obtain larger amounts of the active fraction. For measurement of n.m.r. spectra solubility factors dictate the use of water or aqueous methanol as solvents. Using Fourier transform (F.t.) n.m.r. and aqueous solutions it is only possible to scan about a hundred times and thus well defined spectra could not be obtained. The



pH7 2000V 45min. scale 1:4

C=coloured material uv=visible under u.v. light

Fig. 4. The chromatogram after electrophoresis of the crude concentrate.

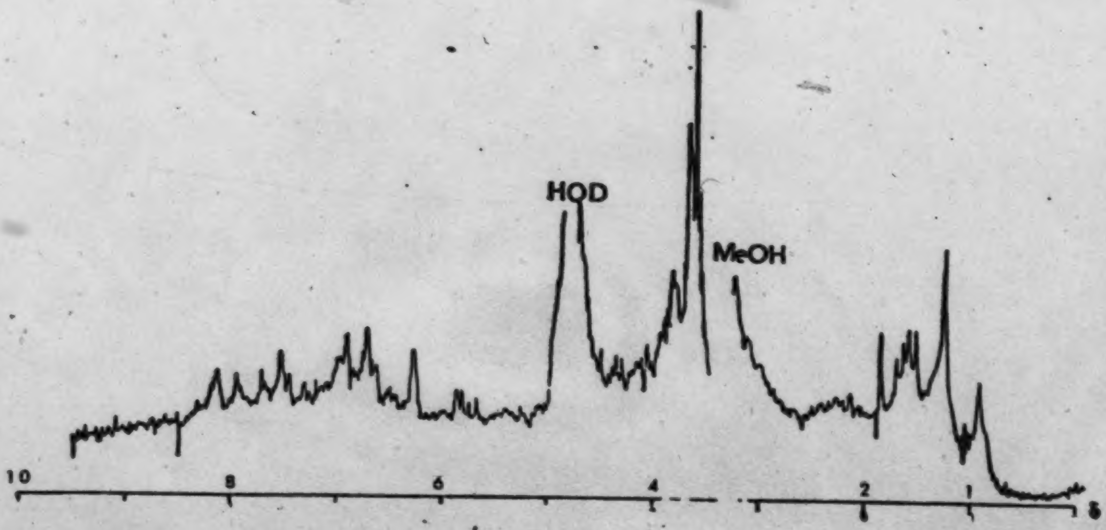
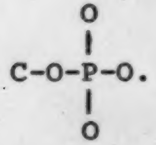


Fig. 5. The p. m. r. spectrum of the active fraction obtained by electrophoresis.

best spectrum obtained is shown in Fig. 5. The aromatic region contains many hydrogen signals spread over a wide range, indicating the presence of heteroatoms. There is a large peak in the region where sugars resonate and a small peak in the range where saturated hydrocarbons resonate. This spectrum suggested that possibly there was a nucleotide or nucleoside-type compound present[33].

Aqueous solvents are very suitable for carbon magnetic resonance spectroscopy since they produce no solvent peak in the spectra. Nevertheless, a c.m.r. spectrum of the crude concentrate could not be obtained. Using F.t. n.m.r. and pulsing the sample more than 60,000 times did not produce any peaks discernable above the noise level of the spectrum.

The crude concentrate was tested for the presence of nucleoside phosphates with ammonium molybdate reagent[34]. The reagent is sprayed on the chromatogram which is left overnight. The paper is then exposed to ammonia fumes; nucleotides appear as blue spots. This reagent also gives a positive test for phosphate buffer. Thus electrophoresis was repeated using sodium citrate and hydrochloric acid buffered at pH7 and similar operating conditions as for the earlier experiments. Using the phosphate reagent described, it could be shown that both inorganic phosphate, i.e., PO$_4^{\equiv}$, and bound phosphate were present as in Fig. 6.

Since the colour tests indicated the presence of phosphate a ^{31}P n.m.r. spectrum of the crude concentrate was determined[35]. The spectrum shows a single unsplit peak. It resonates at the same chemical shift as the phosphorus of adenosine monophosphate and is well shifted from the position of the resonance of inorganic phosphate, PO$_4^{\equiv}$[36]. This indicates that the environment of the phosphorus is  $C-O-\underset{O}{\overset{O}{P}}-O$. Only a single peak is seen in the ^{31}P n.m.r. spectrum showing a single environment for the phosphorus but the chromatogram from electrophoresis shows the presence of more than one phosphorus compound. It is probable that hydrolysis or decomposition occurs during electrophoresis. This would account for the lowered activity of the material obtained after electrophoresis.

Having demonstrated the presence of phosphorus the crude concentrate was examined for the presence of nitrogen bases.* A sample was hydrolyzed with 72% perchloric acid

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* Work done in conjunction with Dr. W. Kokke.

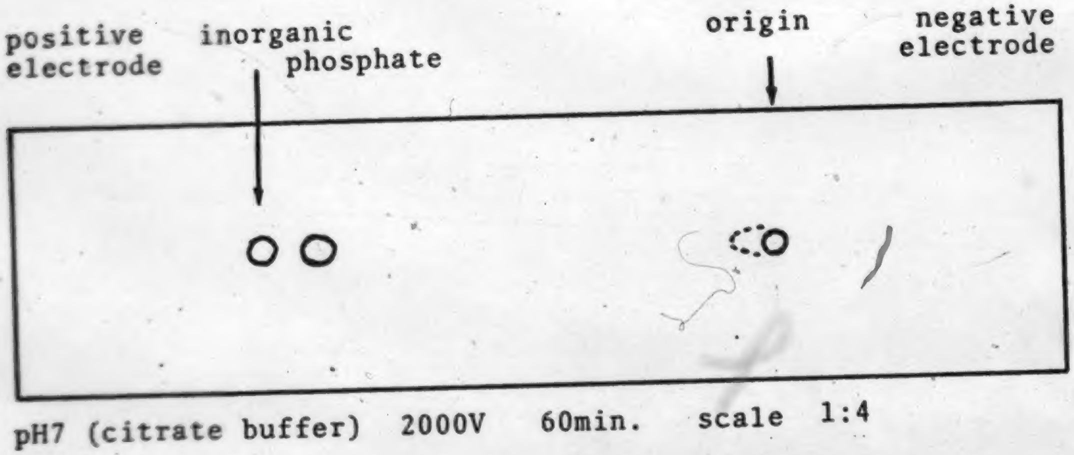


Fig. 6. The chromatogram after electrophoresis of the crude concentrate showing the presence of phosphorus compounds.

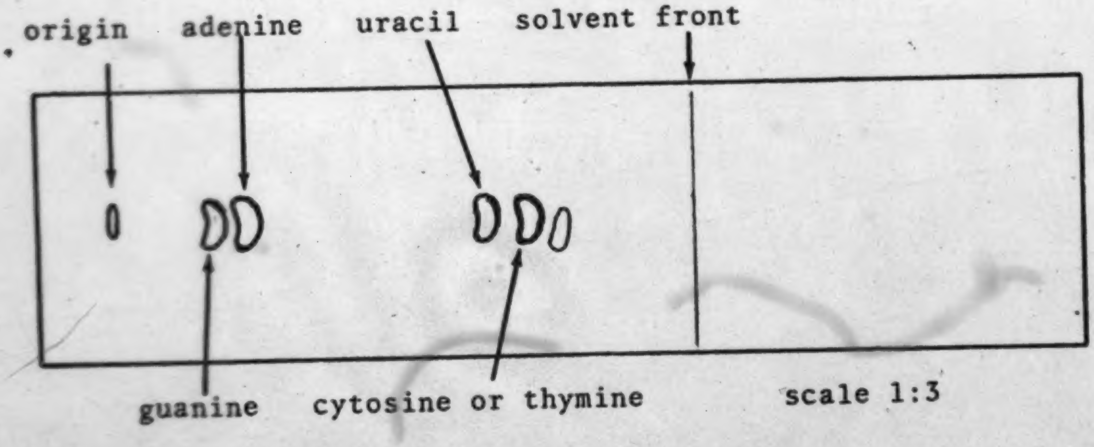
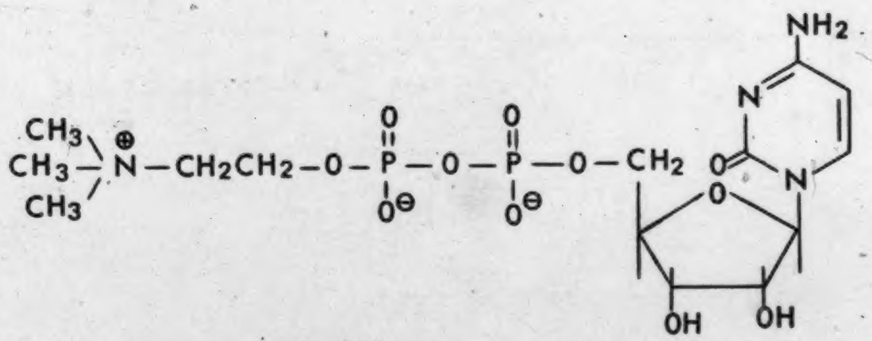


Fig. 7. The paper chromatogram of the product of hydrolysis of the crude concentrate.

for two hours[37]. Paper chromatography of the product[38] showed the presence of guanine, adenine, uracil and a fourth compound, not positively identified but possibly thymine or cytosine, Fig. 7. In addition there was a large amount of unidentified material which remained at the origin.

Hence there is nucleotide-type material present in the crude concentrate. The c.d. spectra of nucleosides have been extensively investigated[39] and nucleosides of the common bases do not have absorptions at wavelengths longer than 300 nm. Hence the 360 nm absorption of the crude concentrate is due either to a persistent impurity or to a modified base.

It seems probable that the nucleotide-type material is responsible for the swimming response of *S. coccinea*. Choline esters of diphosphonucleotides, *e.g.*, (*3*), are naturally occurring compounds[40] and structures of this type have many properties common to those described for the active component. Work is continuing to determine the nature of the nucleoside material and to confirm whether this is indeed the active compound.



(3)

EXPERIMENTAL

Melting points were determined on a Fischer-Johns hot-stage melting point apparatus and are uncorrected.

High pressure liquid chromatography was performed using a Waters Associates Liquid Chromatograph Model ALC/GPC-301.

Infrared spectra were recorded on a Perkin-Elmer Model 421 dual grating infrared spectrophotometer.

Proton magnetic resonance spectra and ^{19}F n. m. r. spectra were measured using a Varian Associates Model HR-100 spectrometer, in the Fourier mode it was interfaced to a Digilab FTS/NMR 3 Data System.

C. m. r. spectra and ^{31}P n. m. r. spectra were determined at 22.63 MHz in the Fourier mode using a Bruker HFX-90 spectrometer in conjunction with a Nicolet-1085, 20 K mercury computer.

Mass spectra were recorded on an A. E. I. Model MS-9 mass spectrometer.

C. d. spectra were determined on a Jasco o. r. d./c. d. SS-20-2 (modified).

U. v. spectra were recorded on a Cary Model 15 u. v. and visible spectrometer.

Electrophoresis separations were done on a Savant Instruments Flat Plate Electrophoresis Apparatus Model FP 22A-S. Whatmann number 2 paper was used for preparative separations. Number 1 paper was used for paper chromatography and small scale separations.

Microanalyses were performed by the Microanalytical Laboratory of this department.

E. s. r. spectra were determined by a Varian Associates Model V 4500 spectrometer at 100 KHz modulation.

Specimens of *Dermasterias imbricata* were obtained by divers from subtidal waters off the coasts of the San Juan Islands, U.S.A. The wet starfish weighs typically around 500 g. and is about 25 cm. across. Specimens used weighed as little as 200 g. and as much as 800 g. *Dermasterias imbricata* of any size will produce a swimming response.

Stomphia coccinea were also obtained from the San Juan coasts, by dredging from depths of about sixty fathoms. They are normally attached to shells of *Modiolus modiolus*. A typical size is three to four cm. tall, a base of five cm., an oral disc of three cm. and tentacles are about 1 cm. long. Most specimens used for tests were in this size range. Some smaller animals were available but were not used since little work has been done to determine if any size-response relationship exists. Generally it appears that small anemones react in the same way as full size ones.

Testing of Solutions

The animals were kept in salt water aquaria at Friday Harbour Laboratories in San Juan. Before being used for testing the anemones were made to swim to *Dermasterias* and then allowed to settle and reattach in a numbered glass petri dish. Any anemones that did not swim fairly readily to *Dermasterias*, and these were very few, were not used in the tests.

All significant testing of the solutions was performed at Friday Harbour Laboratories. At the start of the work it was assumed that anemones could be kept in tanks at the University of Alberta and used for testing purposes. Although the anemones survive for long periods, as much as a year, in such tanks, they very quickly (within two weeks) become useless for testing purposes. Their sensitivity to active extracts decreases very rapidly and when they do swim it usually takes much longer and is a much more sluggish motion than found with fresh animals. Animals kept at Friday Harbour Laboratories have a continual supply of fresh sea water and remain useful for long periods.

A test is carried out by carefully lifting an open anemone on a petri dish from the water. If this is done carelessly the anemone usually closes up. Some of the solution to be tested, usually three drops, is placed on the tentacles. Generally no contact is made between the tentacles and the dropper but in any case the results appear to be independent of whether there was contact or not. The anemone is observed out of the water for sixty to ninety seconds, during which time period practically all the responses occur. This method was found to be more reliable than underwater testing. Very little test solution is required and dilution problems are avoided.

Four stages are recognised in a swimming response: 1) the anemone rises up, its column extended, with concurrent shrinking of the basal disc; 2) the oral disc opens and the tentacles are spread like a flower; 3) there are rocking or sudden whip-like motions; 4) there is a complete detachment of the basal disc as shown in Fig. 8*. In response to a very active solution these four stages can follow each other very closely, some occurring simultaneously, and be complete in as little as six seconds. In a fast response the animal usually detaches before it undergoes any rocking motion and continues its rocking motion for several minutes afterwards, hence the "swimming motion". In response to a dilute or low activity solution the four stages can be clearly separated and the rocking motion generally precedes

* Photos kindly supplied by Dr. D. Ross.

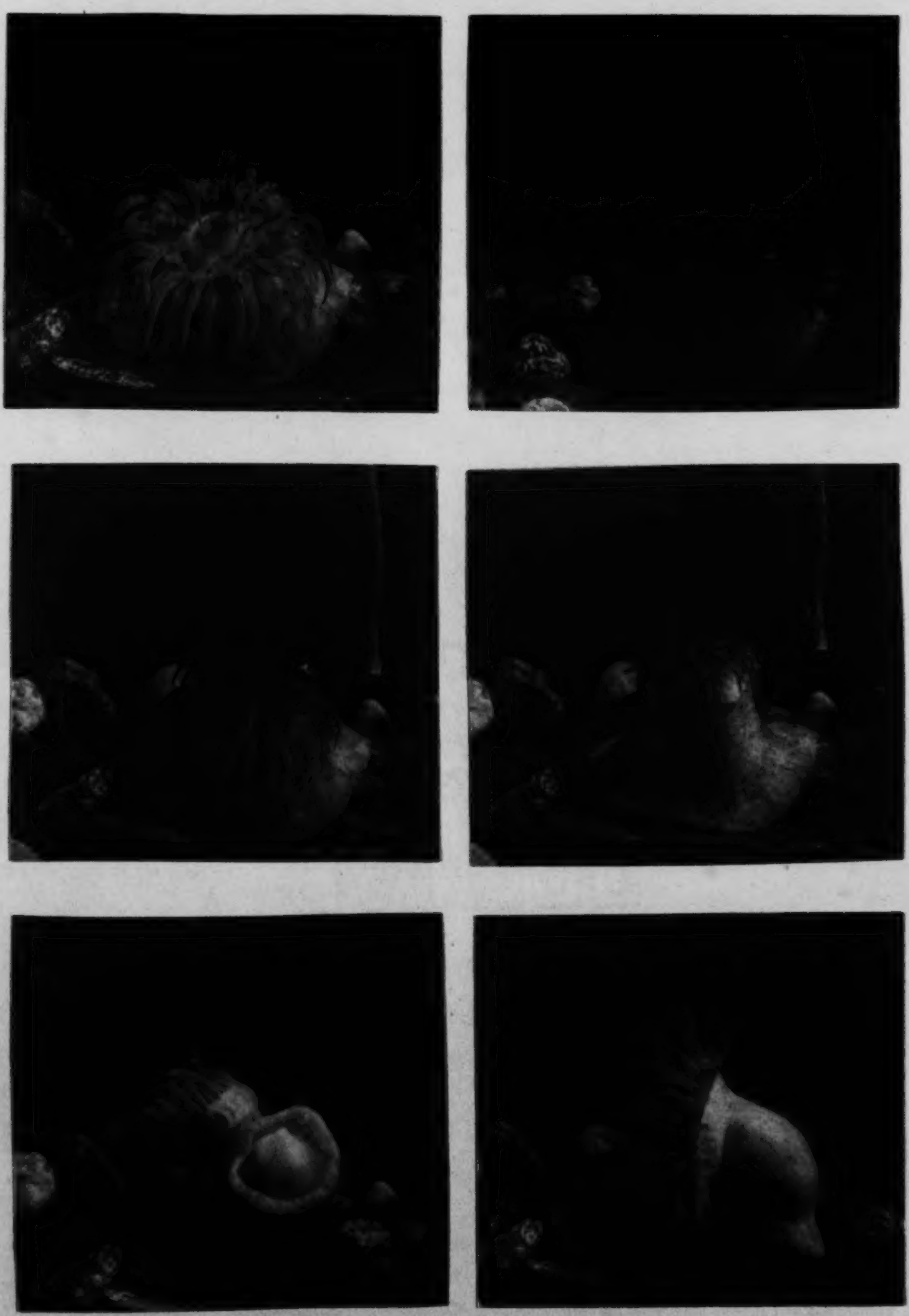


Fig. 8. The swimming response of Stomphia Coccinea.

detachment. Detachment may take up to sixty seconds after application of the test solution and in these cases there will usually be less rocking motion after detachment. In very few cases is detachment observed after sixty seconds.

There is considerable difficulty associated with many biological tests resulting from the fact that animals, even actinaria, are individuals and often behave in an individual way, thus sometimes producing inconsistent results. An important factor in the response of the anemone appeared to be the condition of the tentacles; if they are "sticky" it shows that they are releasing nematocysts. It was observed that if the tentacles of the anemone are not sticky then it was much less likely to swim to active solutions or to Dermasterias. It has been observed that after application of a nutrient the anemone will no longer swim to Dermasterias rubbings or extracts, possibly for similar reasons[41].

Another factor appeared to be that the longer an anemone had remained fastened to its substratum the less likely it was to swim in a test. Hence at the end of each day's testing all the anemones that had not swum were made to do so by contact with Dermasterias. In this way a certain uniformity could be produced in the condition of the anemone.

Each solution was tested on five S. coccinea. A solution that is definitely active will make two or more of the five anemones swim. A low activity solution will produce rocking and possibly one swimming response from five anemones. If a solution produces less than three rocking responses it was regarded as inactive. Swimming and rocking are responses to definite stimuli, they cannot be produced by merely taking the anemone from the water or by application of sea water or distilled water.

At each stage of the purification a check was made to determine if more than one component was producing the swimming response. This was done by recombining all the fractions from the particular separation and testing it on the anemones. At no time was any increased activity found in a recombined sample when compared to the most active fraction.

Isolation of Active Material

The weighed starfish was cut into quarter inch slices and shaken for an hour with distilled water (2 ml./g. of starfish). The water was decanted and added to the coelomic fluid. The starfish pieces were then rinsed with acetone, to remove water, and shaken with chloroform (2 ml./g of starfish). The chloroform was decanted, the pieces rinsed again with acetone and the whole procedure repeated two more times.

The aqueous extracts are combined to give a yellow solution which quickly turns mauve and then dark brown. The solution is filtered through celite and evaporated to dryness under high vacuum. The black powder obtained is extracted once with chloroform to remove traces of chloroform soluble material. This black powder is active at dilutions of 0.5 to 0.1 mg./ml. of distilled water.

Isolation of Steroids (in conjunction with Dr. David King)

The acetone fractions contained almost no water soluble material and were added to the combined chloroform extracts and the mixture evaporated under reduced pressure to give a thick red oil.

A preliminary separation using a florisil column and elution with Skellysolve B, carbon tetrachloride, benzene and chloroform, yielded the crude steroids in the benzene fraction. The steroids were purified by column chromatography over alumina, eluting with diethyl ether.

High pressure liquid chromatography indicated that two steroids were present and these were separated using a five-foot by quarter inch i.d. corasil column, toluene solvent and a flow rate of 14 ml./hr.

The two steroids were shown to be lathosterol, m.p. 121 - 122° C, and cholesterol, m. p. 148° C, by comparison with authentic samples. The abundance of the two compounds was in the ratio 39:2 respectively.

Isolation of the "Crude Concentrate"

A 40 cm. by 2.5 cm. diameter Sephadex column was prepared from one hundred grams of Sephadex G15. Two millilitres solution of the total aqueous crude, containing 500 mg. of solid were applied to the column and eluted with distilled water. The flow rate was adjusted to about 30 ml./hr. and 10 ml. fractions were collected. Black foamy material eluted in the ninth, tenth and eleventh fraction. Sugars eluted in the thirteenth fraction and a dark yellow band eluted in the eighteenth fraction. The active material eluted between the twentieth and seventieth fraction. This volume of 500 ml was evaporated to dryness under high vacuum to give a dark brown powder, weight approximately 20 mg. This powder is active at dilutions of 0.04 mg./ml to 0.01 mg./ml.

This material was involatile and no mass spectrum could be obtained. The p.m.r. spectrum shows a single sharp peak at δ 3.60 (Fig. 1). The c.d. of this material shows absorptions at 255, 300, 318 and 370 nm. as shown in Fig. 2. Its i.r. spectrum (nujol mull) shows bands at 3300 (broad), 2900, 1650 (very broad), 1050 cm^{-1}.

Combustion analysis of the crude concentrate showed 30% C, 4% H, 8% N and no chlorine, iodine or sulphur. Fluorine was not detected by ^{19}F n.m.r. By X-ray fluorescence spectroscopy 0.5% Br was detected in the total crude and 0.05% in the active crude. However after electrophoresis (see below) the active fraction did not contain any bromine, all of it was detected in the inactive fractions. E.s.r. spectroscopy failed to detect any species with unpaired electrons, e.g., transition metals.

Silylation of Crude Concentrate

The crude concentrate (2 mg.) was stirred overnight in 2 ml. of hexamethyldisilylamine, $[(CM_3)_3Si]_2NH$ at room temperature then concentrated to dryness. Virtually none of the resulting material was soluble in acetone or chloroform.

Acetylation of Crude Concentrate

The crude concentrate was stirred with acetic anhydride (2 ml.) and pyridine (5 drops) at room temperature for twenty-four hours. This was concentrated to dryness under high vacuum. Virtually none of the resulting material was soluble in acetone.

Methanol Fractionation

The crude concentrate (100 mg.) was shaken in one litre of methanol and filtered. The resulting solution was concentrated in stages and the precipitated material filtered off at each stage (summarized below). The minimum concentration, to produce swimming activity, of each fraction was:

Fraction A. insoluble in 1000 ml., 34 mg., concentration > 0.1 mg./ml.

Fraction B. insoluble in 30 ml., 6 mg., concentration $\leqslant$ 0.02 mg./ml.

Fraction C. insoluble in 15 ml., 6 mg., concentration $\geqslant$ 0.1 mg./ml.

Fraction D. insoluble in 3 ml., 2 mg., concentration $\geqslant$ 0.1 mg./ml.

Fraction E. insoluble in 0.5 ml., 8 mg., concentration $>$ 0.1 mg./ml.

Fraction F. soluble in 0.5 ml., 40 mg., concentration $>$ 0.1 mg./ml.

The c.d. spectrum of the second fraction, B, showed only two absorption bands as shown in Fig. 3. The intensity of these absorption bands decreased as the solubility increased.

Acid Treatment of Crude Concentrate

The crude concentrate (1 mg.) was dissolved in 5% hydrochloric acid, (1 ml.) and after four hours at room temperature the solution was concentrated to dryness under high vacuum. The resulting powder produced no swimming responses.

Base Treatment of Crude Concentrate

The crude concentrate (1 mg.) was dissolved in 5% sodium hydroxide solution (1 ml.) and after four hours the solution was neutralised with sodium hydrogen sulphate. The powder obtained after concentration of the water under reduced pressure produced no swimming responses.

Electrophoresis of the Crude Concentrate

Whatman no. 2 paper was used for preparative scale work, usually twenty centimetre width for 20 mg. of crude concentrate. A 0.05 M buffer at pH 7 (NaH_2PO_4/Na_2HPO_4) was used, and the paper was dipped in this buffer. Typically a p.d. of 2000 volts for forty-five minutes was used. Where phosphate was unsuitable the buffer used was 0.05 M sodium citrate with sufficient hydrochloric acid to bring the pH to 7.0. After electrophoresis the paper was allowed to dry and then cut into strips. The paper was extracted with distilled water (about 2 ml./$cm.^2$) by leaving the paper in water overnight and then filtering. The solution was then concentrated to dryness under high vacuum. If the typical conditions of 2000 V for 45 minutes were used the material front is about 15 cm. from the origin and the active material is located in the section between 1 cm. and 5 cm. from the origin.

The c.d. spectrum of this active fraction shows absorption bands at 255 and 360 nm. The p.m.r. spectrum (D_2O/CD_3OD) is shown in Fig. 5. A ^{13} c.m.r. spectrum could not be obtained from this fraction.

Methanol was used to extract the active component instead of water on one occasion and the material obtained was also active. The minimum dilution of the active fraction obtained by aqueous extraction was approximately 0.1 mg./ml.

Hydrolysis of the Crude Concentrate (in conjunction with Dr. W. Kokke)

The crude concentrate (25 mg.) and 72% perchloric acid (0.25 ml.) were heated at 100° C for two hours. To this was added 0.25 ml. water and 0.5 ml. 6N potassium hydroxide. The black aqueous suspension was centrifuged and the resulting black supernatant subjected to paper chromatography.

Whatman no. 1 paper was used for chromatography and water (31 ml.), isopropanol (136 ml.) concentrated hydrochloric acid (33 ml.) as the developing solvent Bases were identified by comparison of R_f values and u.v. luminescence with authentic samples. The chromatogram obtained is shown in Fig. 7.

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